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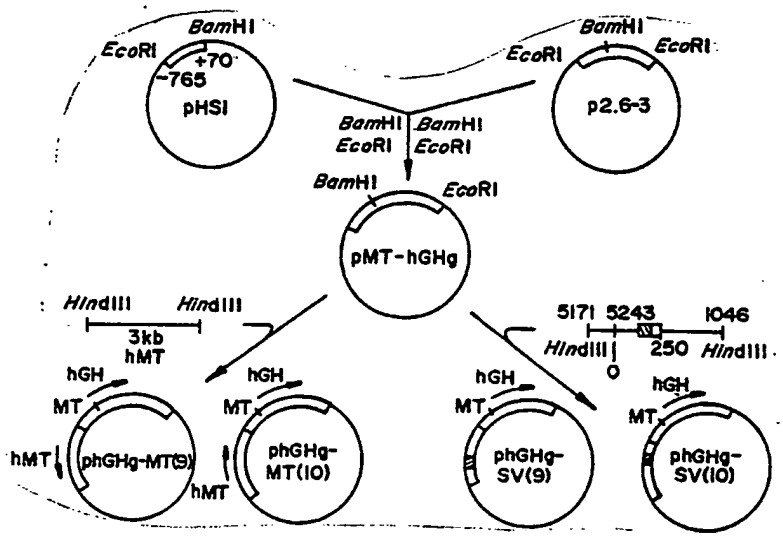

(57) Abstract

An expression system for recombinant production of a desired protein comprises CHO cells transformed with a DNA sequence having the desired protein coding sequence under control of the human metallothionein-II promoter. The cells can be maintained on serum-free medium and induced in the presence of an induction mediator. In addition, the system may include an enhancer element and/or a resistance-conferring gene to provide increased levels of expression. The system can process genomic as well as intronless DNA, and is capable of producing proteins which have the same characteristics as those obtained from native sources. Human growth hormone which is indistinguishable from that produced by the pituitary has been thus produced. Expression systems containing genes encoding alveolar surfactant protein (ASH), apolipoproteins AI and AII (apoAI and apoAII), atrial natriuretic factor (ANF), erythropoetin (Epo), and renin are also contemplated.

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HUMAN METALLOTHIONEIN-II PROMOTER IN MAMMALIAN EXPRESSION SYSTEM

Technical Field

The present invention relates to the expression of desired gene sequences in mammalian expression systems. In part, it relates to a system which utilizes the human metallothionein-II promoter, Chinese hamster ovary (CHO) cellular hosts, which are capable of viability on protein-free medium, and induction in the presence of an induction mediator using relatively non-toxic metals such as zinc ion. Additional improvements in mammalian cell based expression include selection of the transformed host cells for cadmium resistance and/or employing gene amplification techniques and including an enhancer operably linked to the regulatory sequences for gene expression.

The expression system results in gene products encoded by a variety of DNA sequences. The gene products are similar to those from native sources. This similarity is most dramatically shown with respect to human growth hormone (hGH), human alveolar lung surfactant (hASP), and apolipoprotein AI (apoAI).

Background Art

Expression of foreign gene sequences using mammalian hosts is, by now, well-known in the art. Mammalian expression systems are often favored because the host cell possesses processing capability which permits modification of gene products, for example by glycosylation or hydroxylation, unlike bacterial, or even yeast systems.

Also, the ability of mammalian systems to secrete certain gene products efficiently into the medium results in easier harvest and purification.

Because the secreted product must be purified from other proteins in the medium, it is clearly desirable to employ hosts capable of growth in defined media -- i.e., media free from added proteins. While most mammalian cell lines require supplements, such as serum proteins, Chinese Hamster Ovary (CHO) cells can be maintained in defined, serum-free and protein-free medium. (See Hamilton, W.G. and Ham, R.G. In Vitro (1977) 13:537-547). CHO cells are also fast growing and well characterized, free of recognizable dangers, and are thus, in addition to their ability to be maintained serum free, ideal hosts for recombinant protein production.

Mammalian expression, of course, requires compatible control sequences. The most commonly employed control sequences, in particular, promoters, have been viral promoters, most prominently the SV40 promoter (see, for example, EPO Publication 108,667, published 16 May 1984; McCormick, et al, Molec Cell Biol (1984) 4: 166-172) or the gene's own promoter, if compatible (U.S. Patent 4,399,216 to Axel, et al). In general, such promoters are less than satisfactory because they cannot be regulated by environmental factors. Absent such control, the linked coding sequences may be expressed at too high or too low a level in the host organism, or at an improper time, and the worker in the art is powerless to control these aspects of expression. Therefore, attempts have been made to utilize mammalian-compatible promoters susceptible to environmental control. Notable among these promoters are the metallothionein promoters, which natively control the expression of metallothionein proteins -- proteins which bind tightly to heavy metals. In their native state, the metallothionein

promoters are induced both by the presence of heavy metals, for example, cadmium or mercury, and also by steroid hormones and related materials, such as dexamethasone or other glucocorticoids. However, it has not been possible to carry out induction in the absence of protein-supplemented media, thus limiting the use of such promoters to conditions where purification of the protein produced is complicated by the presence of large amounts of additional contaminants.

Various members of the metallothionein promoter family have been used to control expression in mammalian systems. For example, PCT application WO 84/02534, published July 5, 1984, to Hamer, et al, discloses the use of the mouse metallothionein-I (mMT-I) promoter to control the expression of human growth hormone in mouse kidney C127 cells (see also Pavlakis, G.N., et al, Proc Natl Acad Sci (U.S.A.) (1983) 80:397-401). Brinster, et al, Nature (1982) 296:39-42, obtained thymidine kinase production in injected mouse embryos under control of the murine MT-I promoter. Karin, M., et al, DNA (1984) 3:319-325; Nature (1984) 308:513-519 have studied transient expression under the control of the human metallothionein-II_A system (hMT-II_A) in NIH-3T3 cells, by fusing the hMT-II_A promoter to the coding sequences of thymidine kinase to generate hMT-TK chimeric genes, and monitoring expression of TK after deletions in the promoter sequences. Induction was obtained using either cadmium ion, or, in some instances, dexamethasone; and the locations of these regulatory sites were determined by deletion studies.

None of the expression systems disclosed, including those utilizing some form of the MT promoter, permit satisfactory culturing and induction of the host to produce the desired gene product in continuous high

yield and in easily recoverable form, free of added serum or other proteins. The expression system of the present invention permits continuous induction of high levels of expression in hosts grown in serum-free medium, and thus permits high levels of protein production and easy purification of secreted products.

In addition to providing non-toxic induction and culture conditions favorable to purification of the desired protein from the medium, it is desirable to enhance the level of production of this protein. Two general approaches relevant to the invention herein have been used to enhance protein production in the past: inclusion of viral enhancers in the expression system and amplification of the expression system to increase copy number.

Enhancers are cis-acting DNA elements which stimulate transcription. Their activity is relatively independent of their 5'-3' orientation, and, while position dependent to some degree, is retained over distances as long as several thousand nucleotides. Enhancers have been identified in a number of viral genomes and in specialized cellular genes, such as those responsible for the production of immunoglobulins. The enhancer used in the illustration below, which is derived from the simian virus SV40, has been characterized in some detail (Gruss, P., et al, Proc Natl Acad Sci (USA) (1981) 78:943-947; Benoist, C., et al, Nature (1981) 290:304-310; Mathis, D.J., et al, ibid, 310-315). Wasylyk, B., et al, Nucleic Acids Res (1984) 12:5589-5608 showed that the 72 bp repeat which is thought to be the essential element of the SV40 enhancer has a biphasic dependence on distance from tandem conalbumin promoters linked to the coding sequence for early T antigen used as a diagnostic for

transcription enhancement. The invention herein, in some of its aspects, utilizes the stimulating effect of enhancer sequences to increase production levels.

Use of the ability of DHFR for selection of transformants and to amplify in response to certain drugs to effect an increase in the production of protein by a co-transforming expression system has been practiced for several years. See, for example Kaufman, R.J., et al, Mol Cell Biol (1985) 5:1750-1759. It is believed that relevant high production of desired protein through selection for increased drug resistance conferred by co-amplification of the desired gene along with DHFR occurs because the DHFR gene and the co-transforming gene are integrated in nearby positions into the chromosome of the host. Further, amplification in response to the drug occurs over distances of approximately 200 kb, thus carrying the co-transforming gene along with the DHFR in making multiple copies.

The MT gene has not been used analogously. While the ability of the MT gene to amplify in response to cadmium ion is known, this has been studied using bovine papilloma virus (BPV), a self-replicating transforming system, and therefore use of this amplification to co-amplify a desired expression system has not been suggested (Karin, M., et al, Proc Natl Acad Sci (USA) (1983) 4040-4044).

Neither the MT gene nor any other gene has been used as a selectable marker when integrated into the chromosome to identify not only successful transformants, but also high level expressors. Cadmium is so highly toxic that only high level expressors of the cadmium resistance-conferring MT gene can survive. High level expressors for the cotransformed sequence correlate with this resistance because due to their

proximity in the chromosome, both genes are affected by local conditions favoring expression. In some aspects, the invention herein employs an expression system which takes advantage of an MT gene co-transformed with the expression system either to select for transformants with high level expression abilities for the desired sequence, or to amplify the MT and expression sequences simultaneously, or both.

The various aspects of the invention for improving the quality and quantity of expression in CHO cell hosts are illustrated below for the production of a variety of proteins. In one such illustration the gene for human growth hormone is employed, as this material is of practical interest, of benefit therapeutically, and has not been produced in natural form according to current methods. Recombinant production of hGH, although commonly done, does not result in a product which is true to the mixture of materials produced by the pituitary. Native hGH is a mixture which contains approximately 10% of a minor form, 20 kD hGH, in addition to 90% of the major, 22 kD, species. Both species are encoded by a single gene and result from translation of two different mRNAs produced by differential splicing of the second intron from the primary transcript (DeNoto, et al, Nucleic Acids Res (1981) 9:3719). Hence, production employing cDNA for hGH (EPO Application 108,667 (supra)) or production employing a combination of cDNA and genomic sequences, but lacking the second intron (Hamer, WO84/02534; Pavlakis, Proc Natl Acad Sci (supra)) is not satisfactory from this viewpoint, since only the 22 kD form is produced. Even less satisfactory are bacterially produced recombinant hGH preparations (Goeddel, D. V., et al, Nature (1979) 281:544-548;

Martial, J. A., et al, Science (1979) 205:602-607) as the hGH thus produced is not secreted into the medium, and at least a major proportion of the hGH produced contains an N-terminal methionine as a result of the recombinant construction and the inability of the host cell further to process the resulting protein.

Significant advantages over, for example, bacterial expression are also seen for hASP. Isolation of hASP from an alveolar proteinosis patient gives a mixture wherein the major species is a 32 kd protein; this indicates that the putatively native form is glycosylated (White, R.T., et al, Nature (1985) 317:361-363). This is also true of canine ASP where similar heterogeneity (28 kd-36 kd) is found. Human cDNA shows an open reading frame encoding 248 amino acids; the sequence beginning at amino acid 21 encoded by this cDNA corresponds to the 22 N-terminal amino acids of the 32 kd protein isolated from lavage fluid. The cDNA has also permitted location of the exon regions of the gene. The cDNA sequence encodes a number of collagen like Gly-X-Y repeats which contain a proline residue. The presence of hydroxyproline in the native sequence indicates these are hydroxylated. Thus, reconstruction of "native" ASP requires at least two post-translational steps available only in mammalian systems -- hydroxylation of the proline residues and glycosylation.

Apolipoproteins AI and AII, found in connection with lipids as carriers in the bloodstream are encoded as preproproteins and secreted in "pro" form. However, the protein associated with phospholipids to generate the stacked disc structure of the lipoprotein fractions is mostly mature protein (Boganouski, D., et al, J Lipid Res (1985) 26:185). In this case, too, post-transla-

tional modifications of which only mammalian cells are capable, are desirable to generate the native functional form.

The present invention provides not only human growth hormone preparations similar to those produced by the pituitary, human ASP preparations similar to those found in lung lavage fluid, and apolipoproteins functionally associated with phospholipids, but also a means for efficient production and recovery of any desired foreign protein under favorable, high production, easy purification conditions.

Disclosure of the Invention

An expression system is provided which permits environmental control of expression for foreign gene sequences in a mammalian host that is capable of processing the gene products, to obtain materials in high yield which are virtually identical to the naturally produced substances. The host systems employed are capable of processing intron sequences, so that genomic coding units may be used directly. They are also capable of glycosylation, hydroxylation, "pro" sequence cleavage, or other protein modification after translation, and of processing normally associated signal sequences so that, if desired, the materials are secreted into the medium under conditions where the medium is free of substantial amounts of contaminating protein. The control sequences respond to inducers of low toxicity in the presence of non-protein induction mediators. In addition, the expression system in some embodiments is capable of selection for high expression and of amplification, and may be further modified with enhancers to elevate the levels of production. Thus, the expression system of the present invention provides

a host capable of post-translational processing, control of expression, expression at high levels, and ease of purification for the protein produced. This combination of advantages and even subsets thereof are unavailable in the expression systems previously known.

In one aspect, the invention relates to an expression system for any desired coding sequence. The system comprises the mammalian metallothionein-II (hMT-II) promoter operably linked to the coding sequence and transformed into mammalian cells maintained on a defined protein-free medium, particularly CHO cells. Optionally, the system further contains an operably linked enhancer capable of elevating the levels of production and/or a resistance conferring gene capable of effecting amplification of the entire system. The system is induced by a mixture of a non-toxic metal ion in combination with an induction mediator such as iron ion or transferrin.

The invention also relates to methods of expressing genes in mammalian host cells using these expression systems, and to the proteins thus produced. It also relates to methods for selecting recombinant cells capable of high levels of expression and for amplifying a desired expression system by taking advantage of co-transformation with an MT gene and treatment with heavy metal ion. Cells chosen for heavy metal ion resistance can express such systems in serum free medium without an added induction mediator.

Another aspect relates to proteins produced in conformity with their native functional forms. In particular, these proteins include human growth hormone recombinantly produced in a form which mimics that natively obtained from the pituitary; hASP which is hydroxylated and glycosylated like that obtained from

lavage fluid and apolipoprotein which are obtained in mature form free of pro sequences. These and other preparations which conform to the native proteins can be produced using the methods and expression systems of the invention.

Brief Description of the Drawings

Figure 1 shows the production of human growth hormone by CBI-37 cells as a function of time, at various zinc ion inducing levels.

Figure 2 shows the construction of pHS1, a host expression vector which permits insertion of a coding sequence under control of the hMT-II promoter and of two additional host vectors: pHS1-MT, which contains an expressible metallothionein gene for amplification; and pHS1-SV40, which contains an operably linked SV40 enhancer.

Figure 3 shows the construction of pMT-hGH, an expression plasmid for human growth hormone, and of the corresponding expression vectors which contain the expressible metallothionein gene (phGH-MT) and the SV40 enhancer (phGH-SV40).

Figure 4 is the DNA sequence and deduced amino acid sequence of a construct including the genomic sequence which encodes human alveolar surfactant protein (hASP).

Figure 5 shows the DNA sequence and deduced amino acid sequence for a construct including cDNA encoding hASP.

Figure 6 shows the insert containing hASP encoding sequences used to obtain pASPcg-SV(10).

Figure 7 shows the sequence of the genome encoding human atrial natriuretic factor (hANF).

Figure 8 shows the construction of the modified Epo gene.

Figure 9 shows the DNA sequence encoding preprorenin.

Figure 10 shows the distribution of hGH production capability of the various colonies derived from pools of CHO cells transformed with pMT-hGH, phGH-MT, and phGH-SV40.

Figure 11 shows the results of polyacrylamide gel electrophoresis on radiolabeled proteins secreted by CBI-25 and CBI-37.

Figure 12 shows a comparison by SDS-PAGE of pituitary-secreted hGH with hGH preparations from CBI-37 cells.

Figure 13 shows SDS-gel results for Endo-F treated and untreated supernatants from ASP-producing transformant A-38.

Figure 14 shows SDS-gel results for Endo-F treated and untreated supernatants from ASP-producing transformant D-4.

Figure 15 shows a polyacrylamide gel of extracts from CHO cells transformed with various apolipoprotein AI (ApoAI) expression vectors.

Figure 16 is an electron micrograph of the complex formed by apoAI from transformants with endogenous lipid.

Figure 17 is an electron micrograph of the complex formed by apoAI from transformants with phosphatidyl choline.

Figure 18 shows an SDS gel of CHO and AtT-20 pMT-PPPRen transfected cell supernatants.

Modes of Carrying Out the Invention

A. Definitions

As used herein, "operably linked" refers to a juxtaposition such that the ordinary functions of the operably linked materials may be carried out. Thus, a promoter "operably linked" to a coding sequence refers to a configuration wherein the coding sequence can be expressed under control of the promoter in compatible hosts.

"Expression system" refers to a collection of components as subsequently designated and may include, as specified, only a coding sequence operably linked to control sequences, to these sequences further linked to an enhancer, to a vector containing these, to cells transformed by the vector, or to cultures containing the transformants, including medium components.

"Defined medium" refers to a culture medium which has no protein-containing supplements. Most mammalian cell cultures require such supplements. Serum preparations, such as fetal calf serum, in amounts on the order of 10% are ordinarily employed.

"Induction mediator" is a material added, in addition to the metal ion inducing the MT promoter, whose presence permits this induction to occur in defined medium. In the context of the expression system of the present invention, it has been found that iron (ion) in concentrations of 1-3 x 10^{-5} M or transferrin at 5 µg/ml or more behave as induction mediators. Iron ion is most conveniently provided as iron (II) since iron (III) tends to precipitate. However, iron (III) can also function as an induction mediator. While the transferrin is an iron-containing protein, the amounts required for its function as an induction mediator are much smaller than those used for protein

supplements. Therefore, it does not interfere with purification and is not considered to be a "protein supplement" so as to cause the medium to cease being "defined". Also, it need not be added until induction is initiated.

"Human metallothionein-II" promoter (hMT-II) refers to control sequences derived from the human MT-II gene or their functional equivalents. The control sequences of this gene are described in detail by Karin, M., et al, Nature (1982) 299:797-802.

"Host cells", "transformant cells", "cell cultures", "cell lines", and so forth, are used interchangeably herein, as will be clear from the context. Host cells suitable for recombinant transformation refer to cells which have been or are intended to be recipients of new DNA sequences, most commonly in the form of plasmids, but including other transferable DNA forms as well. In this regard, these terms refer not only to the immediate recipient, but also its progeny. Progeny includes product cells of cell division, as well as of other reproductive mechanisms. Progeny includes cells which contain substantially identical DNA sequence content, as well as those wherein the DNA has been altered by accidental or deliberate mutation. It is understood that such mutations may occur as a matter of course in the production of progeny. All progeny which maintain the functionality of the initial transformant, most commonly the ability to produce a specific desired protein, are included in this definition.

"Chinese Hamster Ovary" (CHO) cells include the standard cell line ATCC CCL-61, and its relatives isolated from the same source tissue, as well as derivatives thereof. Derivatives are mutants of the

line which may differ genotypically or phenotypically from the original line, but which are obtained therefrom by intentional or inadvertent mutation.

Similarly AtT-20 cells include ATCC cell line CCL-89 and its relatives and derivatives as described above.

"Derived from" as it pertains to, for example, DNA or protein sequences refers to similarity in structure and not necessarily to physical derivation.

"Amplifiable toxin-resistance conferring gene" refers to a DNA sequence capable of producing (in suitable hosts) a protein which confers resistance on the host to an otherwise toxic element placed in the host's environment and which gene may be produced in multiple copies in the presence of this toxic element. "Toxin" here refers to any material which is deleterious to the host at the concentration levels supplied. Appropriate exemplary toxins are drugs such as methotrexate, to which DHFR confers resistance, and Cd^{+2}, to which the MT protein confers resistance. Both the DHFR and MT genes may respond to the appropriate toxin by amplification, i.e., production of multiple copies.

B. General Description

The present invention offers a substantial improvement in recombinant protein production by virtue of combining particularly favorable conditions for expression in mammalian cells. The metallothionein-II control sequences are advantageously used by virtue of their ability to be inducible by zinc ion, as opposed to the mercury or cadmium ion induction required for MT-I. The latter metals are much more highly toxic to cells, and do not permit, therefore, finely tuned regulation of

expression based on regulation of the inducing ion concentration level. In addition to hMT-II illustrated below, MT-II control sequences isolated from other mammalian species may be used. While hMT-II is preferred, MT-II from, for example, bovine, equine, monkey, hamster, porcine, or murine sources is satisfactory. The MT-II genes from mouse, hamster and monkey have been isolated. See, e.g., Searle, P.F., et al, Mol and Cell Biol (1984) 4:1221-1230.

In the expression system of the invention, these control sequences are used in cells which are maintained on defined media. An integral feature of the system is addition to the defined medium, as induction mediators, 1-3 x 10^{-5} M iron salts or 5 µg/ml or more of transferrin. These additions permit the control sequences to be induced under defined medium conditions. The use of defined medium is a tremendous advantage in production of desired proteins, in that the ability to provide culture conditions wherein the medium does not contain extraneous protein, such as is usually supplied by serum, permits the desired protein to be secreted into a contaminant-free environment.

A useful host expression vector as a component of the expression system is one which contains restriction sites downstream of the MT-II control sequences to permit insertion of a desired coding sequence. Such a vector, illustrated below by pHS1, contains replication sites operable in bacteria, to permit amplification of the host vector and coding sequence insert fragments. The coding sequences ligated into the restriction sites may be either cDNA sequences or genomic fragments containing introns, as the resulting vectors are compatible with hosts capable of processing introns.

Additional improvements may be made to the expression systems by including an operably linked enhancer and/or a toxin-resistance conferring gene, which can select for efficient expression and can stimulate amplification of associated sequences.

Enhancers act in a "cis" manner, and thus need to be included on the same DNA as the remainder of the expression system. Any enhancer sequence from viral or from specialized cells of higher organisms may be used. Viral enhancers have been disclosed which are derived from retroviral long terminal repeats, polyoma virus, Bk virus, and adenovirus, as well as from SV40 as illustrated below. Certain specialized cellular expression systems such as those for ovalbumin and immunoglobulins may also contain enhancer sequences. These enhancers appear to exert their most dramatic effects on relatively weak promoters, and their ability to impact expression controlled by the strong MT-II promoter is surprising. As used in the systems of the present invention, the enhancer sequences are ligated within several kb of the promoter regulating the expression of the desired coding sequence, and in either orientation with respect to it. Preferable orientations and distances for the SV40 enhancer are disclosed hereinbelow in connection with the illustrated systems; these preferences are variable depending on the enhancer and regulated system selected. It is believed that for most systems, the SV40 enhancer is most advantageously located 250-400 bp upstream of the start of transcription.

Genes conferring resistance to toxins can be used to select transformants which are effective in expression of foreign, transforming DNAs. The DNA of the expression system for the desired protein can, of

course, be associated with the resistance gene by ligating it into the same transfer vector, but this is not necessary in all cases. Since transforming DNA, even from multiple vectors, is often integrated into the genome of its mammalian host to reside in proximal positions, even resistance genes introduced on co-transforming plasmids can be used to select for increased expression of the desired gene. Indeed for cells transformed with modified pHS1 vectors, integration is required as the vector is not self-replicating in mammalian hosts. Any toxin-resistance conferring gene may be used, such as the genes encoding DHFR or MT. The resistance gene preferred in the present improved expression system is the metallothionein gene, which confers resistance to the toxic effects of heavy metals such as cadmium. By selecting transformants in medium containing low levels of cadmium -- i.e., up to about 5 μM, individual clones can be selected which have acquired resistance to this toxin concentration, possibly by synthesizing multiple copies of the MT gene, but in any case, by being more effective in producing the metallothionein protein. In such clones, there is a high probability that co-transformed sequences will have been amplified or reside in a more favorable expression environment as well, and hence will have an increased ability to produce the proteins encoded by them. The cell lines which have been selected on this basis, as illustrated below, appear to retain toxin resistance even in the subsequent absence of selective pressure.

Certain resistance-conferring genes, including the MT gene, respond to the presence of the toxin by producing multiple copies both of themselves and of associated DNA. Therefore, in addition to using the MT

sequences for selection, advantage is taken of the ability to coax gene amplification in selected cells. This procedure, as outlined, for example, in Kaufman, et al (supra), consists of protracted subculturing in incrementally higher levels of the toxic agent to obtain progressively higher levels of gene copy number. Because of the association between the MT gene and the desired expression system in the transformants, multiple copies of the expression system are obtained using this technique.

The expression vectors, according to the invention, are transformed into host cells which can be maintained on defined medium, although they may have been grown in the presence of serum. Appropriate cells include CHO cells and their derivatives, although any cell line capable of such serum-free maintenance may be used. The transformation may be carried out using a co-transformed antibiotic resistance marker plasmid, either along with or instead of the amplifiable toxin-resistance conferring gene which may also be used as a marker with desired higher expressing cells selected simulataneously. Replication in the host relies on integration of the appropriate sequences into the host genome in the illustration below, but self-replicating vectors may also be used.

The transformed cells are selected for either co-transforming antibiotic resistance or for toxin resistance conferred by a co-transforming amplifiable toxin resistance conferring gene or both. The selected cells are grown in suitable medium, and this growth medium may, if desired, contain serum or other protein supplements prior to induction. (If an amplifiable resistance-conferring gene is included in the system, the transformed cells may also be amplified to high copy

number by growing the transformants in increasing concentrations of toxin as described above.) Since the induction of the MT-II promoter is controllable, the medium can be and is exchanged for a defined medium upon induction. Efficient growth is thus permitted while retaining the advantage of a simplified secreted protein purification.

It is of course possible to induce the cells directly in the medium upon which they are grown, using zinc ion in the concentration range of 1-5 x 10^{-4} M, approximately. (This concentration range has no definite limits, but amounts within the foregoing arbitrarily set limitations are workable, apparently with an optimum of around 3 x 10^{-4} M zinc ion concentration.) However, such direct induction is disadvantageous from the viewpoint of the subsequent protein purification, and it is preferable to exchange the serum-containing medium for basal medium lacking the protein supplement.

Therefore, in the preferred procedure, which is distinctive to the herein invention, this medium replacement is made. The inducing zinc ion (in the range of about 2 x 10^{-5} - 2 x 10^{-4} M) is added along with an induction mediator selected from the group consisting of iron ion and transferrin, an iron-containing protein. When the induction mediator is transferrin, the amount added should be about 5 µg/ml. Larger amounts can be used, but, of course, are disadvantageous as they add contamination to the medium when protein purification is to be done. The amount of iron ion required, preferably as iron (II), is between 1-3 x 10^{-5} M iron. The basal medium itself contains approximately 3 x 10^{-6} M iron; however, this is insufficient to permit induction to take place in the

absence of serum. Concentrations greater than about 3 x 10^{-5} M iron result in precipitation of the iron at the pH under which the cells are grown. Transformation with the MT gene and subsequent selection for heavy metal resistance results in cultures which do not require and added induction mediator.

Relevant data showing suitable ranges of zinc ion concentration are shown in Figure 1, which illustrates the effect of various concentration levels on hGH production according to the protocol of example, D.5.a, below.

After induction, the desired coding sequence is expressed under the control of the MT-II promoter in copious amounts and, if provided with a leader sequence operable with the mature protein sequence in mammalian cells, is secreted into the medium. While it is possible to produce proteins internal to the cellular environment using the expression system of the invention, there is no particular advantage in doing so, and the leader sequence ordinarily associated with most secreted mammalian proteins is usually included in the desired coding sequence. In addition, while cDNA sequences may also be used in the expression systems, the ability of this host to utilize and express genomic sequence containing introns is advantageous, and can sometimes be particularly important in permitting production of proteins which mimic those naturally produced.

Any desired coding sequence may thus be expressed using the system of the invention. Natively secreted proteins containing their native leader sequences may be produced to effect secretion of the recombinant form; however, proteins which are normally not secreted may also be provided, using recombinant

techniques, with leader sequences compatible with the mammalian cell hosts and with their own amino acid sequences or can be produced intracellularly.

Exemplary of the proteins which may be produced using the method of the invention are the hormones, such as human growth hormone or other mammalian growth hormones, insulin, auriculin, and the like, useful for correcting metabolic defects; viral proteins, such as those encoding capsid proteins for hepatitis or foot-and-mouth disease viruses, useful for the production of vaccines; lymphokines, such as lymphotoxin, the interleukins, or the interferons, useful in therapy; or other miscellaneous proteins, such as urokinase, tissue plasminogen activator, or alveolar surfactant protein, useful in the treatment of specified conditions.

The foregoing is, of course, simply an illustrative, very partial list. The coding sequence for any desired protein is workable in the system of the invention.

C. Standard Methods

The methods to obtain coding sequences from cDNA or genomic libraries are generally known in the art and details are available from standard reference sources. These techniques are used to obtain desired coding sequences for insertion into the vectors of the invention. In addition, many desired coding sequences have already been cloned and are obtainable either by oligonucleotide synthesis techniques or from existing vectors. For those sequences which are not thus available the procedures of ¶s C.1 and C.2 may be used.

C.1. cDNA or Genomic Library Production

Human genomic libraries are constructed in λ phage as is known in the art. See, e.g., Maniatis, T.,

et al. Cell (1978) 15:687-701. Alternatively, double-stranded cDNA can be synthesized from mRNA isolated using standard techniques, and prepared for insertion into a plasmid vector such as pBR322 using homopolymeric tailing mediated by calf thymus terminal transferase (Sutcliffe, J.G., Nucleic Acid Res (1978) 5:2721-2732). First strand cDNA is synthesized by the RNA-dependent DNA polymerase from Avian Myeloblastosis Virus, by priming with oligo (dT) 12-18 on 5 µg mRNA. The RNA template is then liberated from the nascent DNA strand by denaturation at 100°C for 5 min, followed by chilling on ice. Second strand DNA is synthesized by using the large fragment of DNA polymerase I of E. coli, relying on self-priming at the 3'-end of the first strand molecule, thereby forming a double-stranded hairpin DNA. These molecules are blunt-ended at the open-ended termini, and the hairpin loop is cleaved open with S1 nuclease from Aspergillus oryzae. S1 nuclease digestion of the double-stranded cDNA takes place in 300 mM NaCl, 30 mM NaOAc, pH 4.5, 3 mM $ZnCl_2$ for 30 min at 37°C with 600 units enzyme. The cDNA is extracted with phenol:chloroform, and small oligonucleotides are removed by three ethanol precipitations in the presence of ammonium acetate. This is done as follows: a half volume of 7.5 M ammonium acetate and two volumes ethanol are added to the cDNA solution, which is precipitated at -70°C. The blunt-ended, double-stranded cDNA is then fractionated by size using gel filtration through a column (0.3 x 14 cm) Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) or by ultracentrifugation in 5-20% glycerol gradient followed by fractionation of the gradient. cDNA roughly greater than the desired length, e.g., 300 base pairs is retained and recovered by precipitation with 70% ethanol. Short (10-30

nucleotides) polymeric tails of deoxycytosine are added to the 3' termini of the cDNA using a reaction containing 0.2 M potassium cacodylate, 25 mM Tris, pH 6.9, 2 mM dithiothreitol, 0.5 mM $CaCl_2$, 200 mM cDTP, 400 µg/ml BSA, and 40 units calf thymus terminal deoxynucleotide transferase for 5 min at 22°C. The reaction is extracted with phenol:chloroform, and small oligonucleotides are removed with three ethanol precipitations in the presence of ammonium acetate.

The tailed cDNA is annealed with a host vector such as pBR322 which has been cleaved with, for example, PstI and tailed with oligo dG. In one operable embodiment 2.5 µg pBR322-dG DNA is annealed with the cDNA at a vector concentration of 5 µg/ml, and the hybrids are transferred into E. coli MC1061 by the $CaCl_2$-treatment described by Casadaban, M., et al, Mol Biol (1980) 138:179-207.

C.2. Probing cDNA or Genomic Libraries

cDNA or genomic libraries are screened using the colony hybridization procedure. Each microtiter plate is replicated onto duplicate nitrocellulose filter papers (S & S type BA-85) and colonies are allowed to grow at 37°C for 14-16 hr on L agar containing 15 µg/ml tetracycline. The colonies are lysed with 10% SDS and the DNA is fixed to the filter by sequential treatment for 5 min with 500 mM NaOH/1.5 M NaCl, then 0.5 M Tris HCl(pH 8.0)/1.5 M NaCl followed by 2 x standard saline citrate (SSC). Filters are air dried and baked at 80°C for 2 hr.

For nick-translated probe, the duplicate filters are prehybridized at 42°C for 16-18 hr with 10 ml per filter of DNA hybridization buffer (50% formamide (40% formamide if reduced stringency), 5 x SSC, pH 7.0,

5x Denhardt's solution (polyvinyl pyrrolidone, plus Ficoll and bovine serum albumin; 1 x = 0.02% of each), 50 mM sodium phosphate buffer at pH 7.0, 0.2% SDS, 50 µg/ml yeast tRNA, and 50 µg/ml denatured and sheared salmon sperm DNA).

Samples are hybridized with nick-translated DNA probes at 42°C for 12-36 hr for homologous species and 37°C for heterologous species contained in 5 ml of this same DNA hybridization buffer. The filters are washed two times for 30 min, each time at 50°C, in 0.2 x SSC, 0.1% SDS for homologous species hybridization, and at 50°C in 3 x SSC, 0.1% SDS for heterologous species hybridization. Filters are air dried and autoradiographed for 1-3 days at -70°C.

For synthetic (15-30 mer) oligonucleotide probes, the duplicate filters are prehybridized at 42°C for 2-8 hr with 10 ml per filter of oligo-hybridization buffer (6 x SSC, 0.1% SDS, 1 mM EDTA, 5x Denhardt's, 0.05% sodium pyrophosphate and 50 µg/ml denatured and sheared salmon sperm DNA).

The samples are hybridized with kinased oligonucleotide probes of 15-30 nucleotides under conditions which depend on the composition of the oligonucleotide. Typical conditions employ a temperature of 30-42°C for 24-36 hr with 5 ml/filter of this same oligo-hybridization buffer containing probe. The filters are washed two times for 15 min at 23°C, each time with 6 x SSC, 0.1% SDS and 50 mM sodium phosphate buffer at pH 7, then are washed once for 2 min at the calculated hybridization temperature with 6 x SSC and 0.1% SDS, air dried, and are autoradiographed at -70°C for 2 to 3 days.

If the amino acid sequence of the desired protein or nucleotide sequence encoding it in mRNA is

known, the DNA for insertion into the host vectors of the invention may be obtained either by synthetic means, or, if vectors containing such sequences are on deposit or available, by cloning such vectors. For synthesis of the coding sequences, alternating sense and anti-sense overlapping single stranded oligonucleotides are prepared, and the alternating sense and anti-sense single stranded portions filled in enzymatically by treating with DNA polymerase and dNTPs. The oligomers are prepared by the method of Efimov, V.A., et al (Nucleic Acids Res (1982) 6875-6894), and can be prepared using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nM substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pM γ32P-ATP (2.9 mCi/mM), 0.1 mM spermidine, 0.1 mM EDTA.

C.3. Modification of Available Coding Sequences

For sequences from cDNA or genomic DNA which require modifications in order to obtain desired mutant proteins, for example, site specific primer directed mutagenesis is used. This is conducted using a primer synthetic oligonucleotide complementary to a single stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top

agar, permitting plaque formation from single cells which harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The resulting plaques are hybridized with kinased synthetic primer at a temperature which permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques which hybridize with the probe are then picked, cultured, and the DNA recovered.

C.4. Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 µg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 µl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous

fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM dTT and 5-10 µM dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or exonuclease Bal-31 results in hydrolysis of any single-stranded portion.

Ligations are performed in 15-50 µl volumes under the following standard conditions and temperatures: 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM dTT, 33 µg/ml BSA, 10 mM-50 mM NaCl, and either 40 µM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 µg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold

molar excess of linkers) are performed at 1 μM total ends concentration.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) in order to remove the 5' phosphate and prevent religation of the vector. Digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^+ and Mg^{+2} using about 1 unit of BAP or CIP per μg of vector at 60° for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/ chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments. The desired sequences are thus recovered from colonies responding to probe.

C.5. Verification of Construction

In the constructions set forth below, correct ligations for plasmid construction are confirmed by first transforming E. coli strain MC1061 obtained from Dr. M. Casadaban (Casadaban, M., et al, J Mol Biol (1980) 138:179-207) or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell, D.B., et al, Proc Natl Acad Sci (USA) (1969) 62:1159, optionally following chloramphenicol amplification (Clewell, D.B., J Bacteriol (1972) 110:667). The isolated DNA is analyzed

by restriction and/or sequenced by the dideoxy method of Sanger, F., et al, Proc Natl Acad Sci (USA) (1977) 74:5463 as further described by Messing, et al, Nucleic Acids Res (1981) 9:309, or by the method of Maxam, et al, Methods in Enzymology (1980) 65:499.

C.6. Hosts Exemplified

Host strains used in cloning and expression herein are as follows:

For cloning and sequencing, E. coli strain MC1061 or HB101 was used.

The cells used for expression are Chinese hamster ovary (CHO) cells which, under the conditions herein described, may be maintained on defined medium.

D. Examples

The examples below are intended to illustrate the invention but not to limit it.

D.1. Construction of Host Expression Vectors

pHS1

The plasmid pHS1 contains 840 bp of the hMT-II sequence from p84H (Karin, M., et al, Nature (1982) 299:297-302) which spans from the HindIII site at position -765 of the hMT-II gene to the BamHI cleavage site at base + 70. Plasmid p84H was digested to completion with BamHI, treated with exonuclease Bal-31 to remove terminal nucleotides, and then digested with HindIII. The desired 840 bp HindIII/blunt fragment was ligated into pUC8 (Vieira, J., et al, Gene (1982) 19:259-268) which had been opened with HindIII and HincII digestion. The ligation mixture was transformed into E. coli HB101 to Amp^R, and one candidate plasmid,

designated pHS1, was isolated and sequenced by dideoxy sequencing. pHS1, as shown in Figure 2, contains the hMT-II control sequences upstream of a polylinker containing convenient restriction sites.

pHS1-SV40

A pair of host expression vectors containing the SV40 enhancer in operable linkage to the MT-II promoter was constructed by inserting an 1120 bp SV40 DNA fragment into the HindIII site preceding the MT-II promoter sequences in pHS1. The SV40 DNA fragment spans the SV40 origin of replication and includes nucleotide 5171 through nucleotide 5243 (at the origin), the duplicated 72 bp repeat from nucleotide 107-250, and continues through nucleotide 1046 on the side of the origin containing the 5' end of late viral mRNAs. This HindIII 1120 bp fragment is obtained from a HindIII digest of SV40 DNA (Buchman, A.R., et al, DNA Tumor Viruses, 2d ed (J. Tooze, ed.), Cold Spring Harbor Laboratory, New York (1981), pp. 799-841), and cloned into pBR322 for amplification. The cloning vector was cut with HindIII, and the 1100 bp SV40 DNA fragment isolated by gel electrophoresis and ligated into HindIII-digested, CIP-treated, pHS1. The resulting vectors, designated pHS1-SV(9) and pHS1-SV(10), contain the fragment in opposite orientations preceding the MT-II promoter, as shown in Figure 2. In pHS1-SV(9), the enhancer is about 1600 bp from the 5' mRNA start site; in the opposite orientation it is approximately 980 bp from the 5' mRNA start site. Both orientations are operable, but the orientation wherein the enhancer sequences are proximal to the start site provides higher levels of expression. It is believed that deletions

which place the enhancer 250-400 bp upstream of the transcription start are optimal.

Additional vectors were constructed which place the SV40 enhancer 3' terminus 190 bp, 250 bp, and 360 bp respectively upstream from the 5' end of the TATA box. The constructions were based on the mapping of the upstream regulatory regions of the human MT promoter described by Karin, M., et al, Nature (1984) 308:513-519. All constructions retain the sequences containing the duplicated sites for regulation by heavy metals, but the constructions with the 190 bp and 250 bp separations do not retain the sequences for glucocorticoid regulation which is further upstream from these sites.

These vectors, designated pHS'-SV190, pHS'-SV250, and pHS'-SV360 are prepared as follows; all constructions are identical except for the length of sequence containing the metallothionein promoter and upstream region which is supplied as a fragment excised from pHS1.

For pHS'-SV190, pHS1 is digested with SacII, blunted, and ligated to KpnI linkers. The DNA is then digested with EcoRI and KpnI to liberate the appropriate portion of the MT-II control sequences. Similarly, for pHS'-SV250, pHS1 is digested with HgaI, blunted, ligated to KpnI linkers and digested with EcoRI and KpnI; for pHS'-SV360, DdeI is used in the initial digestion.

An intermediate vector containing the SV40 enhancer is prepared by inserting the HindIII/KpnI fragment of SV40 (which extends from position 5171 to position 294 and which contains the enhancer element 50 bp from the KpnI site) into KpnI/HindIII digested pUC19 to obtain pUC-SV. (pUC19 contains three convenient restriction sites in the polylinker region, in order,

HindIII, KpnI, and EcoRI.) The finished vectors are obtained by inserting the KpnI/EcoRI fragments prepared as described above into KpnI/EcoRI digested pUC-SV.

pHS1-MT

Host expression vectors containing both the MT-II promoter (to which the desired coding sequences will be ligated) and the entire MT gene were constructed by inserting the 3 kb HindIII fragment containing the entire human metallothionein-II gene (Karin, M., et al, Nature (1982) 299:297-802) into HindIII-digested pHS1. The resulting plasmids, pHS1-MT(9) and pHS1-MT(10), contained the entire metallothionein gene 5' of the promoter in each of the two possible orientations. See Figure 2.

These vectors are useful as host vectors to construct expression vectors for any appropriate gene; however, alternate constructions of the desired expression vectors can be utilized, as described below, for, for example, the hGH-encoding sequences.

D.2. Construction of MT Vector for Co-transformation

To permit selection or amplification using different starting levels of the expression system and the MT gene conferring cadmium resistance, general shuttle vectors designated pUC9/MT were constructed by ligating the MT-II gene obtained as a HindIII fragment from p84H (supra) into HindIII-digested, alkaline phosphatase-treated, pUC9.

D.3. Construction of Expression Vectors

The basic expression vector for a DNA-encoding protein X under the control of the MT-II promoter is designated pMT-X. Those vectors in which pMT-X is

modified also to contain operably linked SV40 enhancers are generically designated pX-SV40. When the enhancer sequences correspond in location to those in pHS1-SV(9) and pHS1-SV(10) -- i.e., 1600 bp or 980 bp from the transcription start site, respectively, the vectors are designated pX-SV(9) or pX-SV(10). Those vectors in which pMT-X is modified to contain the complete MT gene are generically designated pX-MT, again pX-MT(9) and pX-MT(10) indicate analogy to pHS1-MT(9) and pHS1-MT(10).

In all cases, when placed after "X" in the designation, "c" refers to a cDNA insert; "g" refers to a gene.

D.3.a. Vectors for the Expression of Growth Hormone

pMT-hGHg

The genomic sequences encoding hGH were isolated from p2.6-3 (DeNoto, et al, Nucleic Acids Res (1981) 19:3719)by digestion with BamHI, which cuts at the 5' end of the first exon, and EcoRI, which cuts 3' of the functional gene, followed by polyacrylamide gel purification. The isolated fragment was ligated into BamHI/EcoRI digested pHS1 and the ligation mixture transformed into E. coli MC1061 to Amp^R. Successful transformants were screened by restriction analysis, and a strain containing the desired plasmid, pMT-hGHg (see Figure 3) was further propagated to prepare quantities of plasmid DNA.

phGHg-SV40

In a manner similar to that described above for constructing pHS1-SV(9) or pHS1-SV(10), but substituting for pHS1, pMT-hGHg, a pair of vectors containing the hGH gene under the control of the MT promoter, and operably

linked to SV40 enhancer, designated, respectively, phGHg-SV(9) and phGHg-SV(10), were obtained. The ligation mixtures were used to transform E. coli 1061 to Amp^R, and the correct constructions verified.

phGHg-MT

Expression vectors which contain the entire hMT gene useful for selection and amplification were obtained by digesting the pMT-hGHg vectors prepared above with HindIII, treating with alkaline phosphatase, and inserting the 3 kb HindIII fragment containing the hMT gene, in a manner analogous to that used in the construction of pHS1-MT. This construction is also shown in Figure 3.

D.3.b. Vectors for Expression of Alveolar Surfactant Protein (ASP)

pMT-ASPg

For the genomic ASP vector pMT-ASPg, the coding sequences were obtained from the phage vector λ:gHS-15, deposited with ATCC on 7 December 1984 and given accession no. ATCC 40146. To obtain λ:gHS15, a human genomic library cloned into bacteriophage Charon 28 (Rimm, D.L., et al, Gene (1980) 12:301-310) was obtained from Dr. T. Maniatis, Harvard University. Approximately 1.5 x 10^6 phage were grown on E. coli K803, and plaque lysates were transferred to nitrocellulose filters as described by Benton, W. D., et al, Science (1977) 196:180-182. The filters were probed with DS-1 cDNA which had been kinased by the nick-translation method of Rigby, P.W.J., et al, J Mol Biol (1977) 113:237-251. Filters were prewashed in hybridization buffer (0.75 M NaCl, 0.75 M sodium nitrate, 40% formamide, 0.05% SDS, 0.02% bovine serum

albumin, 0.02% Ficoll - 400,000, 0.02% polyvinyl pyrollidone, 0.1% sodium pyrophosphate, 50 µg/ml yeast tRNA, 50 µg/ml denatured sheared salmon sperm DNA) at 42°C for 1 hr. 5 x 10^5 cpm probe was added per ml fresh hybridization buffer and the filters were incubated in this buffer at 37°C for 16 hr. They were then washed in 0.45 M NaCl and 0.045 M sodium citrate and 0.1% SDS two times at 50°C, and exposed for autoradiography overnight. Six potential clones containing sequences hybridizing to DS-1 cDNA were purified. The most strongly hybridizing clone, gHS-15, was characterized.

A 700 bp EcoRI fragment from gHS-15 hybridized with the DS-1 probe and was chosen for sequence analysis. This EcoRI fragment was purified, inserted into M13mp9, sequenced and found to be extensively homologous with the corresponding canine sequence.

The entire human coding region was contained within two contiguous BamHI fragments: a 5' 1.2 kb and a 3' 3.5 kb fragment. Both BamHI fragments were individually subcloned into the BamHI site of M13mp8 and sequenced. Additional fragments were similarly sequenced according to the strategy shown in Figure 3. The sequence information was analyzed using various Intelligenetics (Palo Alto, CA) computer programs in accordance with the instructions of the manufacturer. The regions containing the signal peptide, precursor sequence and mature apoprotein were identified by comparison to the canine ASP cDNA. From the sequence analysis, the 5' terminus of the gene is encoded within the 1.2 kb BamHI fragment and the 3' terminus within the 3.5 kb BamHI fragment. The gene is interrupted by three introns at positions 1218 bp, 1651 bp and 2482 bp, with

position 1 being the first bp of the 1.2 kb BamHI fragment.

The entire ASP coding sequence is excisable from λ:gHS-15 by digestion with BamHI as two fragments, 1.2 kb and 3.5 kb, respectively. pHS-1 was cleaved with BamHI, and the two foregoing BamHI fragments from λ:gHS-15 were ligated into the site. The ligation mixture was transformed into E. coli MC1061 to Amp^R, and successful transformants screened by restriction analysis. The strain containing the desired construction with the coding sequence of ASP under the control of the hMT-II promoter was designated pMT-ASPg, and propagated to prepare quantities of plasmid DNA.

pMT-ASPg-Apo

For the genomic DNA-containing vector having a downstream Apo sequence, pMT-ASPg-Apo, the coding sequences were obtained as an HinfI/EcoRI fragment of the gene extending from nucleotide 950 to nucleotide 3432, containing exons 2, 3, and 4, and part of exon 5 (White, R.T., et al, Nature (1985) 317:361-363). This fragment was ligated to a 500 bp fragment from the 3' end of the human ApoAI gene (Shoulders, C.C., Nucleic Acids Res (1983) 11:2827-2837) which contains the polyadenylation signal and polyadenylation site. This construction is as follows:

A derivative of the pMT, pMT-Apo, containing C-terminal regulatory signals was prepared. pMT-Apo harbors a portion of the human liver protein $ApoA_1$ gene (Shoulders, C. C., et al, (supra)) which contains the 3'-terminal regulatory signals. A PstI/PstI 2.2 kb fragment of $ApoA_1$ gene (blunt ended) was cloned into the SmaI site of the pMT polylinker region, and the majority of the $ApoA_1$ gene removed by digestion with

BamHI, blunt ending with Klenow, digestion with StuI, and religation. The resulting vector contains roughly 500 bp of the $ApoA_1$ gene from the 3' terminus as confirmed by dideoxy-sequence analysis.

The 3.5 kb BamHI fragment (above) was truncated at the 3' terminus by digestion with EcoRI (position 3434) and filled in with Klenow. This truncated fragment was cloned, along with the 1.2 kb fragment (above) that had been truncated with HinfI into the BamHI site of pMT-Apo to give pMT-ASPg-Apo. The entire ASP-encoding genomic insert terminated by Apo is shown ligated to the MT-II promoter in Figure 4.

It was expected that this vector would produce a protein 23 amino acids longer than the native preprotein (which includes the signal sequence). The construct lacks exon 1 and therefore translation probably initiates at the ATG beginning at nucleotide 987 of the genomic sequence complementary to native preprotein mRNA, which nucleotide normally resides in the first intron. In the production of native preprotein, exon 1 is spliced to exon 2 at nucleotide 1022, deleting this start codon, and permitting translation to initiate at nucleotide 1046. However, the additional residues do not appear to interfere with secretion, and the normal mature protein is secreted from cells expressing this modified form of the gene.

pASPc-SV(10)

The coding sequences for ASP were inserted into a modified form of the host vector pHS1-SV(10) which contains the enhancer elements proximal to the MT-II promoter region. First, the 500 bp apoAI fragment was inserted into pHS1-SV(10) by isolating this fragment, obtained by digestion of pMT-Apo (described above) and

ligating the isolate into EcoRI/BamHI digested pHS1-SV(10). pMT-Apo was digested with BamHI, blunted, and ligated to the cDNA sequences obtained from pHS10-5 (White, R.T., et al, Nature (1985) 317:361-363) as a blunted EcoRI digest. The cDNA fragment extends from the EcoRI linker joined to the 5' untranslated region to the naturally occurring EcoRI site in the 3' untranslated region (900 bp). The relevant nucleotide sequences are shown in Figure 5, where the starred amino acids represent differences in the primary amino acid sequence from that of the protein obtained from pMT-ASPg-Apo. (The differences result from base changes between human cDNA and the genomic sequences.) Initiation of translation is at nucleotide 56, as in the native sequence.

pASPcg-SV(10)

An additional modification was prepared by integrating pASPc-SV(10) and pMT-ASPg-Apo sequences. Plasmid pASPc-SV(10) was digested with BamHI and EcoRI, and the isolated larger fragment ligated to the 3' portion of the ASP gene obtained by BamHI/EcoRI(partial) digestion of pMT-ASPg-Apo. This represents the portion of the human ASP gene beginning at nucleotide 1154 and extending to nucleotide 3432, this being ligated to the ApoAI gene fragment as above. This construct results in a protein identical to that obtained from pMT-ASPg-Apo, but different at amino acid positions 25, 30, and 34 from that obtained from pASPc-SV(10). The nucleotide sequence of the relevant insert is shown in Figure 6.

D.3.c. Vectors for Expression of Apolipoproteins

pMT-AIg and pMT-AIIc

The apolipoprotein AI (apoAI) gene was isolated from pPSA1.2, which contains the entire coding sequence of the apoAI gene as an insert into the PstI site of pBR322, by digestion with PstI followed by polyacrylamide gel purification. This fragment was originally derived from the recombinant phage, λAI-12 as a 2.2 kb PstI fragment (Seilhamer, J.J., et al, DNA (1984) 3:309) and extends from the 5' untranslated region through the entire coding sequence including introns and terminates beyond the polyA addition site. The PstI insert was blunted by treating with T4 DNA polymerase in the presence of dCTP, ligated into SmaI-digested, BAPped pHS1, and the ligation mixture transformed into E. coli 1061 to Amp^R. The correct construction of the resulting vector, pMT-AIg, was confirmed by restriction enzyme digestion analysis.

In a similar manner, a cDNA sequence encoding human apoAII was cloned into EcoRI-digested pHS1 to obtain an expression vector for apoAII designated pMT-AIIc. The EcoRI insert encoding apoAII was obtained from a human fetal liver cDNA library in λgt10 prepared as described by Huynh, V.T., et al, DNA Cloning Techniques: A Practical Approach (IRL Press, Oxford, 1984), which had been probed with a 45 bp oligonucleotide encoding amino acid residues 140-164 of human apoAII (Sharpe, C.R., et al, Nucleic Acids Res (1984) 12:3917-3932). Of 750,000 recombinants, 10 positive colonies were obtained, and one of these, designated λAII, had a 440 base EcoRI insert corresponding to the full sequence apoAII cDNA, plus about 20 bases 5' of the untranslated region. This

EcoRI insert was used for insertion into pHS1 to obtain pMT-AII.

pAIg-SV40

Expression vectors containing the SV40 enhancer in both orientations operably linked to the genomic sequences encoding apoAI were constructed using SmaI-digested, alkaline phosphatase-treated pHS1-SV(9) and pHS1-SV(10) and inserting a 2.2 kb PstI fragment containing most of the apoAI gene obtained as a PstI digest of pPSA1.3 (described by Seilhamer et al, DNA (1984) 3:309; and by Protter, A., et al, DNA (1984) 3:449). The 2.2 PstI fragment obtained from the digest was isolated and blunted using Klenow for insertion into the SmaI-cleaved host vectors. The resulting ligation mixtures were transformed into E. coli to Amp^R and the correct constructions of pAIg-SV(9) and pAIg-SV(10) were confirmed.

D.3.d. Vectors for Expression of Atrial Natriuretic Factor (ANF)

Atrial natriuretic factor is a protein which regulates the excretion of sodium by the kidneys, and thus controls one of the factors responsible for maintaining proper fluid balance. It is produced in heart atrial cells as a preproprotein and then secreted and processed.

For insertion of the genomic sequence encoding human prepro-ANF, pHS1 was first modified by inserting into the BamHI site, a 24 base BamHI linker fragment isolated from M13mp7, containing convenient restriction sites, including an AccI site. The modified pHS1, designated pHS1', thereby contains this convenient AccI

site helpful in the insertion of the ANF coding sequences.

The desired expression vector, designated pMT'-ANF, was constructed by treating pHS1' with AccI and EcoRI, and then with alkaline phosphatase, and inserting a 2016 bp AcyI(partial, blunted)/EcoRI fragment purified from pHGRB-1, which was described in U.S. Serial No. 622,639, filed 20 June 1984, assigned to the same assignee and incorporated herein by reference. This insert contains the entire ANF gene. The relevant DNA sequence is shown in Figure 7.

D.3.e. Vectors for Expression of Erythropoietin

The gene sequence encoding erythropoietin (Epo) was prepared as follows: The human genomic library, as prepared by Lawn, R.M, et al, Cell (1978) 15:1157-1174 in λ-Charon 4A, was probed with the two 24-mers: 5'-TCTGTCCCCTGTCCTGCAGGCCTC-3' and 5'-CTGGGCTCCCAGAGCCCGAAGCAG-3' designed to be complementary to the exons of the Epo gene (according to Jacobs, et al, Nature (1985) 313:806-810). A hybridizing phage containing a 15 kb insert was thus obtained, and digested with BamHI and EcoRI to obtain a 4.8 kb fragment which contained the entire coding region of the Epo gene, in addition to 200 bp of the 5' untranslated region and the entire 3' untranslated region. This fragment was isolated on polyacrylamide gel, blunt ended, and ligated into the SmaI site of SmaI-digested, CIP-treated, pUC9 for amplification. The BamHI/EcoRI insert from this cloning vector was then isolated and used to supply the Epo coding sequences in the following vectors:

pMT-Epo

pMT-Epo was prepared by cleaving pHS1 with BamHI and EcoRI and inserting the Epo coding sequences as a BamHI/EcoRI fragment above.

pEpo-SV(10)

In a similar manner, pHS1-SV(10) was digested with BamHI and EcoRI and the Epo-encoding fragment inserted to provide the SV40 enhancer in proximal operable linkage to the MT promoter.

pEpo-MT

Similarly, but substituting for pHS1-SV(10), pHS1-MT, the desired vector pEpo-MT containing the entire metallothionein gene as an amplification regulator was obtained.

pEpo'-SV(10)

pEpo-SV(10) was modified to provide, insofar as permitted by the desired coding sequence, a gene having a Kozak concensus sequence at the 5' end and a polyadenylation site at the 3' end. The Epo gene described above lacks the consensus polyadenylation site thought to be required for efficient transcription.

pEpo-SV(10) was digested with ScaI which cuts 7 bases following exon I in the first intron. The resulting blunt ended site was ligated to the oligomer

5'-GATCCAAGATGGGGGTGCACGGTGAGT-3'
GTTCTACCCCCACGTGCCACTCA

to replace the missing portions of the gene N-terminus, to provide the concensus sequence, and to provide a BamHI site at the upstream end. The ligation mixture was treated with BamHI (which removes the 5' untranslated regions of the gene from the end of the

vector), and religated to obtain an intermediate vector containing the Epo gene with a modified 5' terminus.

For the 3' end modification, phGHg-SV(10) was digested with BamHI and SmaI and treated with CIP to provide a host vector lacking the coding sequences for hGH. This linearized vector was ligated to a BamHI/NcoI fragment from the intermediate vector prepared above containing the 5' modified Epo gene lacking most of the 3' untranslated region to provide pEpo'-SV(10) which contains the desired modified gene. The construction is outlined in Figure 8.

D.3.f. Vectors for Expression of Renin

The preparation of a preprorenin expression vector wherein the preprorenin sequences are under control of the MT-II promoter has been described in U.S. Serial No. 719,414 , filed 3 April 1985, and incorporated herein by reference. This description is fully set forth as follows: The DNA encoding preprorenin and its processed products is obtained by probing a cDNA library constructed from kidney cell mRNA. However, it would not be necessary to repeat this procedure, as the sequence is now known, (see Figure 9) and cells harboring one vector containing the complete coding sequence of human prorenin preceeded by a modified human signal sequence are deposited at the American Type Culture Collection. CBI-2B5, harboring pPP14 was deposited with ATCC 26 March 1985 and has accession no. CRL 8758. Methods to obtain entire gene sequences, once the desired sequence is known are now available. See, e.g., Edge, M.D., et al, Nature (1981) 292:756; Nambiar, K.P., et al, Science (1984) 223:1299; or Jay, E., et al, J Biol Chem (1984) 259:6311.

A cDNA library was prepared from oligo dT primed polyA$^+$ RNA, purified from the kidney of a human accident victim. The cDNA library was constructed in the bacteriophage vector λgt10 and probed with appropriate fragments of Charon-4 human renin genomic clones which had been obtained from a human genomic library by probing with mouse submaxillary gland renin cDNA fragments. The library was prepared by standard procedures to obtain double-stranded cDNA from the mRNA, blunt-ending the cDNA with DNA polymeraseI (Klenow), adding commercially available EcoRI linkers, cleaving with EcoRI, and ligating the fragments into EcoRI-digested λgt10 vectors. Two positively responding cDNA clones, together, when sequenced using the dideoxy method of Sanger (supra), were shown to contain the entire renin encoding sequence and 3' untranslated region except for a missing 7 base pairs at the 5' end of the signal sequence. The two clones include 1211 bp of coding region, followed by the entire 3' 198 bp untranslated region followed, in turn, by a polyA tail. The assignment of the sequence to the appropriate preprorenin codons and reading frame was made by comparison to the published Imai, et al (see Figure 9) sequence. Two cloned fragments result because of the unique EcoRI site at position 742 as shown in Figure 9.

The cDNA sequences encoding the preprorenin protein were transferred into pUC9 as the two EcoRI inserts to obtain the cloned vectors pHR1 and pHR2. pHR1 contains the 5' portion and pHR2 contains the 3' portion. pHR1 and pHR2 were prepared by excising the cDNA from λgt10 using EcoRI and ligating each of the resulting fragments into EcoRI-cleaved pUC9.

To construct pPP14, pMT.PRO was used as a host expression vector. pMT.PRO is similar to pHS1 and contains 840 bp of the hMT-II sequence from p84H (Karin, M., et al, Nature (1982) 299:297-802) which spans from the HindIII site at position -765 of the hMT-II gene to base +70. pMT.PRO contains this hMT-II fragment but further includes the ATG start codon when ligated into pUC8 (Vieira, J., et al, Gene (1982) 19:259-268). Thus to construct pMT.PRO, the hMT-II sequence was excised from pMTII-BPV (Karin, M., et al, Proc Natl Acad Sci (USA) (1983) 80:4040-4044) as a HindIII/HindIII fragment, which was then digested with BamHI to obtain the promoter and 5' transcript portion. The HindIII/BamHI fragment containing the hMT-II sequences was then inserted into HindIII/BamHI-digested pUC8 to obtain pMT.PRO.

To construct pPP14, pMT.PRO was digested with BamHI, filled in using DNA polymerase (Klenow) in the presence of the four dNTPs, and then digested with EcoRI. EcoRI cuts immediately downstream from the unique BamHI site which follows the ATG start codon. The 5' portion of the preprorenin gene was excised from pHR1 by EcoRI digestion, and ligated under sticky-end conditions to the EcoRI tail of the opened pMT.PRO vector. Approximately 50% of the inserts will be in the correct orientation. The ligation mixture was then treated with DNA polymerase I (Klenow) in the presence of the four dNTPs, to blunt the remaining unligated EcoRI site. Finally, the vector was recircularized by ligation, and the mixture transformed into E. coli MC1061 to Amp^R. Colonies containing the correct construction, with the 5'-portion of the preprorenin coding sequences in reading frame with the ATG provided by the hMT-II promoter region and 5' transcript, were

picked and cultured. Construction of the intermediate plasmid was confirmed by restriction analysis and sequencing.

The resulting intermediate plasmid seemed unable to accept the EcoRI-excised 3' portion obtained from pHR-2 in the correct orientation. This problem was overcome by replacing the backbone vector of the intermediate, which had been derived from pUC8, with the corresponding sequences from pUC9. To do this, the intermediate vector was treated with HindIII and EcoRI, and the fragment containing the hMT-II promoter/preprorenin 5' portion inserted into HindIII/EcoRI digested pUC9. The resulting modified intermediate was then digested with EcoRI and ligated with the EcoRI/EcoRI fragment isolated from pHR2 to generate pPP14, which contains the entire preprorenin coding sequence.

Since the junction region between the ATG start codon of the pMT.PRO and the 5' terminal portion of the preprorenin encoding sequences were joined by blunt-end ligation after repair of the BamHI and EcoRI cleaved sites, respectively, the sequence in the junction region is 5'-CCATGGATCAATTCCGATGG, thus placing the underlined ATG start codon shown in reading frame with the remainder of the coding sequence. The overlining indicates the junction of the filled in BamHI and EcoRI sites. Thus the coding sequence for the N-terminus of the preprotein of the signal sequence is Met-asp-gln-phe-arg-trp, which contains two additional amino acids in comparison to the native corresponding N-terminal sequence, which is Met-asp-gly-trp.

This vector, designated therein pPP14, is renamed in the present application for consistency, pMT-PPRen.

D.4. Generation of Mammalian Transformants

Each of the vectors described above was transformed into CHO cells as follows: Chinese hamster ovary (CHO)-K1 cells were grown on medium composed of a 1:1 mixture of Coon's F12 medium and DME21 medium with 10% fetal calf serum. The competent cells were co-transformed with the vector of interest and pSV2:NEO (Southern, P., et al, J Mol Appl Genet (1982) 1:327-341). pSV2:NEO contains a functional gene conferring resistance to the neomycin analog G418. In a typical transformation, 0.5 µg of pSV2-NEO and 5 µg or more of the expression vector DNA are applied to a 100 mm dish of cells. The calcium phosphate-DNA co-precipitation according to the protocol of Wigler, M., et al, Cell (1979) 16:777-785, was used with the inclusion of a two minute "shock" with 15% glycerol in PBS after four hours of exposure to the DNA.

Briefly, the cells are seeded at 1/10 confluence, grown overnight, washed 2x with PBS, and placed in 0.5 ml Hepes-buffered saline containing the $CaPO_4$•DNA co-precipitate for 15 min and then fed with 10 ml medium. The medium is removed by aspiration and replaced with 15% glycerol in PBS for 1.5-3 min. The shocked cells are washed and fed with culture medium. Until induction of MT-II-controlled expression, the medium contains F12/DMEM21 1:1 with 10% FBS. A day later, the cells are subjected to 1 mg/ml G418 to provide a pool of G418-resistant colonies. Successful transformants, also having a stable inheritance of the

desired plasmid, are then plated at low density for purification of clonal isolates.

D.5. Assay for Production Levels of Desired Protein

The transformants are assayed for production of the desired protein, first as pools, and then as isolated clones in multi-well plates. The plate assay levels are somewhat dependent on the well size -- e.g. results from 24 well plates are not directly comparable with those from 96 well plates. Clones which are found by plate assay to be producing the protein at a satisfactory level can then be grown in production runs in roller bottles. Typically, the levels of production are higher when the scale up is done. However, there is not an absolute correlation between performance in the plate assay and in roller bottles -- i.e. cultures which are the best producers in the plate assay are not necessarily the best after scale-up. For this reason, typically 100-200 or more individual clones are assayed by various screening methods on plates and 5-10 of the highest producers are assayed under production conditions (roller bottle).

D.5.a. Production of hGH

Plate Assays

Small amounts of the isolates from cells transformed with the hGH-encoding plasmids were grown in multi-well plates after exposure to 10^{-4} M zinc chloride for convenient assay of growth hormone production. Growth hormone determinations were made by standard radioimmunoassays using commercially available reagents (Hybritech, Inc.). Two of 60 clonal isolates from pMT-hGH transformants (CBI-25 and CBI-37) produced large amounts of the desired hGH. CBI-37 was deposited

with the ATCC on 7 February 1985 and given Accession No. CRL-8721.

For cells transformed with phGH-MT(9) or phGH-MT(10), additional steps for cadmium resistance selection were employed to select appropriate transformants. A pool of G418-resistant cells were seeded at 1/10 confluence and grown in the presence of 5 x 10^{-5} M zinc chloride and various amounts of cadmium chloride. A similar pool of transformants carrying pMT-hGH were used as controls. No surviving colonies were obtained at concentrations as low as 2.5 µM Cd^{+2} when the pMT-hGH pool was used for seeding. However, more than 500 colonies survived in media seeded with phGH-MT transformants at 2.5 µM Cd^{+2}, about 100 colonies at 5 µM Cd^{+2}, and 2 colonies at 10 µM Cd^{+2}. No colonies survived at 20 or 50 µM Cd^{+2}. The 10 µM Cd^{+2} resistant colonies were discarded because of abnormal morphology, but colonies from the 2.5 µM and 5 µM Cd^{+2} were grown to confluence to create new pools for comparison of hGH production levels with unamplified transformants.

Pools of transformants were induced for hGH production to obtain comparisons of production levels with and without enhancers or Cd^{+2} selection. The cells were seeded into 5% serum with G418 in 6-well plates using 25 µl cells from confluent 100 mm plates. Two days later, the cells were washed with PBS and refed with serum-free buffer containing 3 x 10^{-5} M $FeSO_4$ and 7 x 10^{-5} M Zn^{+2}.

Transformants with pMT-hGHg showed production levels of 1.4 µg/ml, as did clones transformed with phGHg-MT not selected for cadmium ion resistance. However, phGHg-MT transformants selected in 2.5 µM or 5 µM Cd^{+2} produced 6.5 and 17.5 µg hGH/ml.

respectively. Cells transformed with phGHg-SV(9) or phGHg-SV(10), respectively, produced 7.0 µg/ml and 17.0 µg/ml hGH. Figure 10 shows a graphic representation of the distribution of hGH production levels for individual clones in the various pools. It is clear that phGHg-SV40 transformants exhibit superior properties to transformants with pMT-hGHg or with phGHg-MT in the absence of cadmium ion. The construction wherein the enhancer element is closer to the transcription start appears to give higher yields.

Clones were isolated from the pool resistant to 5 µM cadmium ion and three of the best colonies were isolated. Cd-5-4, Cd-5-12, and Cd-5-15 when grown under production conditions (see below) yielded approximately 120 µg/ml of hGH. When assayed under plate assay conditions, Cd-5-12 yielded 21.5 µg/ml and Cd-5-15 yielded 27.8 µg/ml hGH; other clones, less successful under production conditions, yielded 50-70 µg/ml. Southern blots confirmed that cells picked for 5 µM cadmium ion resistance contained about 20 copies of the expression vector, while the pool which was not cadmium-selected contained an average of 1-3 copies.

The Cd-5-15 clone was cultured and further selected for cadmium resistance at higher levels. The cells were seeded in 15 ml medium containing DME21/Coon's F12, 10% FCS, pen/strep, G418, 50 µM zinc ion and 25 µM cadmium ion in a T-75 flask, and grown to confluence. Cells were then used to seed a 10 cm plate containing 10 ml of the same medium at low density, and single cells were identified microscopically, marked, and picked 2 weeks later into individual wells of a 6 well plate containing the same medium. The ability of the 6 colonies to produce hGH was assayed by seeding at 1/10 confluence into 24 well

plates and assaying growth on days 2 and 3 after transfer. One of the six colonies, designated CB515-25A showed normal growth and gave production levels of hGH at 13 µg/ml and 22 µg/ml after 2 and 3 days growth respectively. CB515-2A was used for roller bottle hGH production as described below.

It was also shown that CB515-25A did not require the presence of cadmium in the medium to maintain production levels. Five equal aliquots of frozen CB515-25A cells were expanded by plating the cells 1:20, then 1:7, then 1:5 into roller bottles (the equivalent of 700 times, the level required in production) under the medium modification conditions shown below and were assayed for hGH production at 1 and 2 days after switch to serum free medium and induction in the presence of zinc as described above. All expansions contained 125 DME121/F12, 10% FCS plus 15 mM Hepes and pen/strep.

Aliquot	Additions	hGH (µg/ml) Day 1	hGH (µg/ml) Day 2	hGH as % of secreted protein Day 1	hGH as % of secreted protein Day 2
1	None	81	133	116	89
2	25 µM Cd^{+2} (+ 50 µM Zn^{+2} preinduction)	92	150	110	91
3	25 µM Cd^{+2}, (first two transfers only) (+ 50 µM Zn^{+2} preinduction)	69	171	73	114
4	G418	69	127	110	85
5	G418 + 25 µM Cd^{+2} (+ 50 µM Zn^{+2} preinduction)	91	153	112	98

These data show that selective pressure is not needed to maintain the ability of the cells to produce hGH at high levels.

An additional set of transformants was obtained by treating the cells with a DNA mixture containing 1 µg PsvII-Neo, 10 µg phGHg-SV(10) and 10 µg pUC9/MT to obtain the results simultaneously taking advantage of cadmium selection and the enhancer activity of the SV40 segment. The transformants were selected in 20 µM cadmium ion and 10 individual clones picked and grown in a 96 well dish. The best of these 10 clones produced hGH at 150 µg/ml.

Production Runs

Standard Induction

The CBI-25 and CBI-37 cells, which had been shown to produce hGH under suitable conditions, were seeded into roller bottles at 1/10 confluence in basal medium supplemented with 10% fetal calf serum, incubated overnight, and then induced for growth hormone production by addition of zinc chloride in the concentration range of 1×10^{-4} M to 3×10^{-4} M. hGH levels rise for 7-10 days, with a final accumulated concentration of 35 mg/l under optimal inducing conditions, 2×10^{-4} M $ZnCl_2$ (see Figure 1).

Figure 11 shows the behavior on gels for ^{35}S-methionine labeled proteins secreted by CBI-25 and CBI-37. After six hours of incubation with 1×10^{-4} M zinc chloride, ^{35}S-methionine was added to the culture, and the proteins secreted into the medium were analyzed by electrophoresis on 15% SDS-acrylamide gels, followed by autoradiography. In Figure 9, lanes 1, 2 and 3 show total secreted proteins from, in order, CBI-37, CBI-25, and untransformed CHO-K1 cells. The

media from the transformed cells show the presence of a major band at approximately 22 kD not present in the untransformed cells. Lanes 4, 5, and 6 show corresponding results for proteins immunoprecipitated with rabbit antiserum to hGH. It is particularly clear from the results in the immunoprecipitate that two major protein bands, corresponding to 22 kD and 20 kD are present. The proportion of the 20 kD band is roughly 10% of the total, corresponding to the second (20 kD) species variant found in growth hormone produced by the human pituitary.

Prolonged exposure of the autoradiograph also shows two minor related species of higher molecular weight, consistent with pregrowth hormone and pre-20 kD growth hormone. The identity of the 22 kD species as mature hGH was further confirmed by comparison with pituitary hGH in reduced and unreduced gel electrophoresis, and by ability to compete with radio labeled hGH for growth receptors on IM9 human lymphocytes (Rosenfeld, R.G., et al, Biochem Biophys Res Comm (1982) 106:202).

Production in Serum-Free Medium

CBI-37 cells were also grown under pilot production conditions and induced in the absence of serum. The cells were seeded into a 490 cm square roller bottle in Coon's F12/DME21, 1/1 medium supplemented with 10% fetal calf serum and 15 mM Hepes. When the cells had reached near confluence (3-4 days), the cells were washed 2 x with Ca^{+2} and Mg^{+2} containing PBS, and the medium was replaced with the same basal medium (serum free and including 15 mM Hepes) containing 6-8 x 10^{-5} M zinc chloride and 3 x 10^{-5} M iron (II) ion, in place of the fetal calf serum

supplement. (It was found that human transferrin at least 5μg/ml could be substituted for the iron ion.) Growth was continued under these conditions by harvesting the medium 2-3 times weekly, and replenishing it with fresh serum-free medium. The secreted hGH was harvested at the rate of 2.5 mg/day. Proportionately more hGH was obtainable from production bottles 2-4 times this size. Production may be extended indefinitely, and has been continued for as long as 8 weeks.

Figure 12 shows the results of SDS-PAGE on proteins from the medium obtained above stained with Coomassie blue. The CBI-37 cell medium shows the presence of hGH species not present in the controls, and purification of the medium proteins with the single step of gel permeation column results in hGH which is approximately 90% pure, as there shown.

Additional similar production runs utilizing a cadmium-resistant transformant containing phGHg-MT (Cd-5-15) and a transformant containing phGHg-SV(10) (V-18) were made for purposes of comparison. The results are tabulated in Table 1 below. Day zero is counted when preinduction medium is discarded and the cells are refed with medium containing 6-8 x 10^{5} M zinc ion, 3 x 10^{-5} M iron (II) and 15 mM Hepes.

Table 1

Day	hGH (µg/ml) CBI-37	hGH (µg/ml) Cd-5-15	hGH (µg/ml) V-18	Accumulated hGH (mg/roller)* CBI-37	Accumulated hGH (mg/roller)* Cd-5-15	Accumulated hGH (mg/roller)* V-18
0	--	--	-	--	--	--
2		94.5	21		11.8	2.6
	28.6			3.6		
4		89.5	38		23	7.4
			32			11.4
6		83.5	36		33.4	15.9
	32.2	56.0	47	7.6	40.4	21.7
8			56			28.7
	33.0		53	11.7		35.4
10		58.5	42		47.1	40.6
	40.3		32	16.7		44.6
12			39			49.5
14	31.6	40.5		22.6	52.2	
16	33.3			26.8		
18	35.6			31.4		
20	30.4			37.5		

*Each roller bottle contains 250 ml medium.

As shown above, the average hGH accumulated per day in the roller bottle is 1.9 mg/day (7.6 µg/ml/day) for CBI-37, 4.4 mg/day (17.6 µg/ml/day) for Cd-5-15, and 4.1 mg/day (16.4 µg/ml/day) for V-18.

Additional production runs were conducted to show that for the Cd-5-15 cells, the addition of induction mediator was unnecessary. The Cd-5-15 cells were seeded into three separate bottles, two 1750 cm^2 and one 490 cm^2, grown to confluence, and then induced exactly as described above except that iron was not included in the induction medium for the 490 cm^2 bottle and for one of the 1750 cm^2 bottles. All showed steady linear production of hGH at approimately

the same rate for a period of 11 days after induction. After 11 days, the comparative amounts of total hGH accumulated (normalized to the 1750 cm^2 bottles) were 93 mg for the bottle containing iron, and 70 mg and 95 mg hGH respectively for the 1750 cm^2 and 490 cm^2 bottles wherein iron was absent. These data show that in the presence of the entire metallothionein gene, the necessity for an induction mediator is obviated.

D.5.b. Alveolar Surfactant Protein Plate Assays

Pools of cells transformed with the various ASP encoding plasmids were grown in multi-well plates and then exposed to 5 x 10^{-5} to 1 x 10^{-4} zinc ion concentration to induce production of ASP. ASP assays were conducted using Western blot employing immunoprecipation with rabbit anti-human ASP polyclonal antiserum followed by ^{125}I protein A and autoradiography.

In more detail, semiconfluent monolayers of individual cell lines growing in McCoy's 5A medium with 10% FBS were washed with phosphate-buffered saline (PBS) and refed with McCoy's containing 10% FBS, 1 x 10^{-4} zinc chloride, and 0.25 mM sodium ascorbate. (Ascorbate may be helpful in mediating the hydroxylation of proline residues.) Twenty-four hours post induction, the cells were washed with PBS and refed with serum-free McCoy's containing the zinc chloride and ascorbate. After 12 hours, the conditioned media were harvested, made 20 mM in Tris, pH 8, and filtered through nitrocellulose in a BRL dot-blot apparatus. The nitrocellulose filter was blocked in 50 mM Tris, pH 7.5, 150 mM NaCl (Tris/salt) containing 5% nonfat dry milk, and then incubated with 1:5000 dilution of rabbit anti-human ASP polyclonal antiserum in the blocking solution, washed several times

in the above Tris/salt, and incubated with 25 µCi of ^{125}I protein A in blocking solution, washed, and autoradiographed.

Most pools transformed with the ASP encoding vectors did not produce ASP detectable in this assay. However, a positive, ASP-secreting cell line, designated A-38, was selected from pMT-ASPg-Apo transformants. In addition, certain pools from cells transformed with pASPc-SV(10), designated ASP-I, or with pASPcg-SV(10), designated ASP-F and ASP-G, produced levels of ASP comparable to those produced by the cell line designated D-4 described below (~2-5µg/ml).

Characterization of ASP Protein

The A-38 cells (supra) were grown to 25% confluence in McCoy's 5A medium containing 10% FBS and then induced with 10^{-4} M zinc chloride in McCoy's containing 10% FBS and 0.25 mM sodium ascorbate. (Half of the cells were also treated with 10^{-6} M dexamethasone.) Twenty-four hours later, the cells were washed with PBS and refed with RPMI medium containing 10% dialyzed FBS, 1 x 10^{-4} M zinc chloride, 0.25 mM sodium ascorbate, and 0.5 mCi/ml ^{35}S-methionine.

Eighteen hours later, the cell supernatant was made 1 mM phenylmethylsulfonylfluoride and immunoprecipitated with rabbit anti-canine ASP antiserum using protein A as carrier. Half of the precipitated protein was boiled in SDS-PAGE sample buffer, and the other half eluted into 0.75% Triton X-100, 0.075% SDS, 0.75% 2-mercaptoethanol, 30 mM EDTA, 75 mM sodium phosphate, pH 1 and incubated for 1 hr at 37° with 0.5 units of endoglycosidase-F (endo-F). Endo-F treated and untreated protein fractions were subjected to SDS-PAGE with the results shown in Figure 13. The Endo-F treated

fraction showed a 30 kD protein (lane F) as compared to 38 kD protein for the untreated (lane E). (Lane M contains size markers, lanes A and B supernatants from untransformed CHO cells, and lanes C and D supernatants from A-38 cells untreated and treated with dexamethasone, respectively.)

Supertransfection to Prepare D-4

An additional cell line, designated D-4, was obtained by supertransfection of A-38 with a mixture of pMT-ASPg-Apo (20 µg) and pSV2:GPT (1 µg). Semiconfluent monolayers of A-38 growing in F12/DMEM21 with 10% FBS were co-transfected, as described above. After 48 hours the cells were split 1:5 into F12/DMEM21 containing 10% FBS and HAT selection drugs. After 17 days of HAT selection, the pool of surviving resistant clones was screened for individual clones producing high levels of ASP by the immunofilter screen method of McCracken, A.A., et al Biotechniques (March/April 1984) 82-87. Briefly, the cells were seeded onto plates at 100 cells per 100 mm dish in F12/DMEM21, 10% FBS. After 5 days (when colonies contain 50-200 cells each), the cells were washed with PBS, refed with serum-free F12/DMEM21, and overlayed with a sterile teflon mesh. On top of the mesh was placed a nitrocellulose filter which was left in place for 8 hr. The nitrocellulose was removed and treated as an immunoblot, first with rabbit anti-canine ASP polyclonal antiserum, then ^{125}I protein A, followed by autoradiography. Of approximately 2000 colonies screened, two gave a detectable signal and one, designated D-4, was shown to express the ASP gene at 10-20 times the level of A-38, or at an amount corresponding to an estimated 2-5 µg/ml ASP.

Characterization

The secreted ASP from the D-4 cell line was isolated from the serum-free medium by affinity chromatography and sequenced at the N-terminus on a gas-phase microsequencer. Determination of a 16 amino acid sequence showed complete homology with the N-terminal portion of the protein isolated from lung lavage; 70% of the total contained an N-terminal Glu residue; the remaining 30% was clipped so as to contain an N-terminal Val (position 2 relative to Glu). This is the same composition as the isolated lavage protein. Hydroxyprolines were present at positions 10, 13, and 16, indicating the ability of the cells to exhibit post-translational processing.

In addition, the protein secreted by D-4 along with the secreted protein fraction from pool ASP-I (supra) and from pool ASP-G (supra) was compared to human proteinosis lung lavage protein using Western blot. Serum-free medium from induced cells was TCA precipitated, treated (or not) with Endo-F and subjected to SDS-PAGE in 12.5% gels. The gel was electroblotted and dot-incubated with rabbit antihuman ASP polyclonal antiserum followed by ^{125}I protein A. The results are shown in Figure 14.

Lanes A and F contain 1 µg alveolar proteinosis protein before and after Endo-F digestion; lanes B, C, and D represent media from D-4, ASP-I pool, and ASP-G pool respectively untreated with Endo-F; lanes G, H, and I represent proteins from these supernatants treated with Endo-F. It is evident that Endo-F treatment reduces the apparent molecular weight of all proteins, and results in more discrete bands.

Production Runs

The supertransfected cell line containing multiple copies of pMT-ASPg-Apo (cell line D-4) was used in a production level run in roller bottles. An 850-cm square roller bottle was seeded with a 10 cm dish containing 2 x 10^6 cells in 10% FCS, 15 mM Hepes, pen/strep, and glutamine. After the cells reached confluence (2-3 days), they were washed 2 x with PBS and replaced with 250 ml of F12/DMEM21, 10 mM Hepes without FCS. The following day the cells were refed with 250 ml of F12/DMEM21, 10 mM Hepes, 5 x 10^{-5} zinc chloride, 10^{-6} M dexamethasone, and 0.25 mM ascorbate. The cells were harvested every 2 days, spun for ten minutes at 1000 rpm, and frozen at -20°C. Production was 1-5 µg/ml/day, assayed by dot-blot Western using polyclonal anti-canine ASP antisera at 1:5000 dilution, as described above. Production drops after about 14-17 days.

D.5.c. Apolipoproteins

Plate Assays

Cells transformed with apolipoprotein expression vectors were verified for production of apolipoprotein by detecting a protein of the correct molecular weight when the cells were permitted to incorporate a ^{35}S methionine.

Transformants, with either pHS1' (as a control) or pMT-AI, were grown to 70% confluence in 9.6-cm square wells in standard medium (RPMI plus 10% dialyzed FBS). The cells were preinduced with 1.5 x 10^{-4} M zinc chloride for 7 hr, at which time 0.15 µg/ml L-[^{35}S] methionine was added. Cells were incubated in the presence of label overnight, and the media harvested. Portions of the media containing 1 x 10^5 cpm were

added to SDS-gel sample buffer and incubated at 100°C for 2 min; second portions (5 x 10^5 cpm) were brought to 1 ml final volumes using 50 mM Tris-HCl, pH 6.8, 0.15 M NaCl, 0.1 mM EDTA, and 2% (V/V) Triton-X 100. Two ml of rabbit anti-human apoAI was added to the suspension, which was then incubated, reacted with protein A sepharose, and washed.

Pools of cells transformed with pMT-AI, as contrasted with pHS1' transformants, show the expected 25 kd band characteristic of the mature native apoAI when run on gels, which band is specifically immunoreactive against human apoAI.

In a similar manner, CHO cells were transformed with pMT-AII, and the production of apoAII protein verified.

To obtain individual colonies from the AI-producing pool, the cells were plated at low density (100-200 cells/ml) in F12/DMEM21 with 10% FBS to produce individual colonies after 4-7 days at 37°C. The colonies were picked and grown to a cell density of about 10^6 cells/ml and then individually assayed for apoAI expression by dot blot Western, using the method of Jahn et al, Proc Natl Acad Sci (USA) (1984) 81:1684-1687.

For apoAI production, the individual colonies were seeded at 25% confluence in 12-well dishes in 1.5 ml F12/DMEM21 plus 10% FBS. After 24 hours the cells were washed with 1 ml PBS and fresh medium containing 1 x 10^{-4} M zinc sulfate was added to begin preinduction. Sixteen hr later the cells were washed twice with 1 ml PBS and refed with 0.65 ml serum-free medium containing 3 x 10^{-5} M zinc sulfate and 3 x 10^{-5} M iron (II) sulfate. After 48 hr of serum-free induction, the media were harvested, centrifuged at 1000 rpm for 5

min to remove all debris, and 0.5 ml of the supernatants applied to a nitrocellulose filter for assay as described by Jahn, et al (supra).

A high-producing apoAI cell line, designated Clone 104, was further verified to produce apoAI incorporating ^{35}S methionine. ApoAI from these cells is produced at a 30-fold higher level than the pool cells and is identifiable in total secreted medium proteins without immunoprecipitation. Clone 104 was deposited with the American Type Culture Collection on 3 October 1985 and has ATCC number 8911.

Effect of Enhancer

Pools of transformed cells were selected and screened for apoAI production using cells transformed with pAIg-SV(9) and pAIg-SV(10). Individual colonies were obtained from these pooled as described above and were cultured and induced for the production of apoAI.

For comparison of expression levels with those of controls (pHS1' and pMT-AI) the individual colonies were subjected to analysis for apoAI in the medium as follows: After the 48 hr of serum-free induction and centrifugation to remove cell debris, protein was precipitated from 0.4 ml samples of the media by the addition of TCA to 10%, and addition of 20 µl bovine insulin as carrier protein. Samples were incubated on ice for 30 min followed by centrifugation at 3000 rpm for 30 at 4°C. The pellets were washed once with 0.5 ml ice cold acetone and then solubilized in 40 µl SDS-gel sample buffer and applied to a 10-20% gradient SDS-PAGE gel according to Laemmli (Nature (1970) 227:680). The gel was stained with Coomassie blue, giving the results shown in Figure 15.

Lane 1 represents the pHS1' control; lane 11 represents cells transformed with pMT-AI; lanes 18 and 19 represent cells transformed with pMT-AI, but grown in roller bottle cultures (which results in higher production); and lane 20 contains molecular weight markers. Lanes 2-10 represent various single colonies of pAIg-SV(9) transformants. Lanes 12-17 represent individual colonies of pAIg-SV(10) transformants. Lanes 2 and 12 represent pools harboring pAIg-SV(9) and pAIg-SV(10), respectively.

It is apparent from Figure 15 that most of the individual colonies produce apoAI at levels higher than the unenhanced vector transformants. The production levels are estimated to be approximately 30 µg/ml.

Production Runs

The pMT-AI-transformed cells designated Clone 104 were grown in 850-cm square roller bottles as described for pMT-ASPg-Apo above. After 2 days of incubation in the original serum-containing medium at 37°C, zinc sulfate was added to a concentration of 1 x 10^{-4} M, and 1 day later the cells were switched to a serum-free F12/DMEM21 medium containing 6 x 10^{-5} M zinc sulfate to induce apoAI production. Two days later the cells were refed with the same protein-free medium but containing 7 x 10^{-5} M zinc sulfate. This is designated "day zero". After day zero, the medium was assayed periodically for apoAI production by centrifugation of a portion of the medium to separate cell debris followed by fractionation of the medium on SDS-gel electrophoresis and staining with Coomassie blue. The stained gels were scanned to quantitate the amount of apoAI protein using purified apoAI standard obtained from Calbiochem (La Jolla, CA). The cells were

refed periodically as above. ApoAI expression increased over the first 6 days from about 10 µg/ml/day at day 2 to 20 µg/ml/day at day 5 and then plateaued at about 30 µg/ml/day.

Characterization and Complexation of ApoAI

The culture supernatants from the production runs in the previous paragraph were shown to be correctly processed and to form lipoprotein complexes similar to those found natively.

The ability of the apoAI to complex with endogenous lipid in the CHO cells was shown as follows: The medium was adjusted to a density of 1.125 g/ml by addition of solid potassium bromide, then centrifuged at 38K rpm for 18 hours in a swinging-bucket rotor. The top fraction was removed by a conventional slicing method, and dialyzed extensively against saline. The material, when examined by SDS gel electrophoresis, gave a prominent band in the 25 kd range, corresponding to apoAI. About 10%-20% of the total apoAI was in the upper fraction, as judged by the relative staining intensities of the 25 kd bands on SDS gels.

Electron micrography of the top-fraction material shows the presence of numerous disc-shape structures characteristic serum of apoAI/phospholipid complexes (described in Hamilton, R.L., et al, J Clin Invest (1976) 58:667-680), as shown in Figure 16 .

The ability of the apoAI to complex with INTRALIPID (Cutter Labs, Berkeley, CA) was shown in an additional protocol. INTRALIPID is an artificial lipid emulsion composed of soybean triacylglycerol and egg lecithins. The mesophase, or phospholipid-rich portion of the emulsion was removed by ultracentrifugal floatation in a discontinuous sucrose gradient. The

bottom layer contained 2 ml of emulsion, 0.6 g sucrose and saline to give a final volume of 4 ml and a density of 1.06 g/ml. This layer, in a polyallomer tube of a Beckman SW41 rotor, was overlayed with 6 ml of an NaCl solution of d = 1.02 g/ml. Finally a third layer was made of 2 ml of saline solution of d = 1.006 g/ml. Centrifugation was at 28,000 rpm at 10°C for 60 minutes.

After centrifugation, the triglyceride rich emulsion on the top of the gradient was separated from the infranatant solution by the tube slicing technique using a Beckman slicer.

The culture medium was concentrated 100 times by ultrafiltration using an Amicon YM 10 membrane. The concentrated medium was incubated with purified INTRALIPID and centrifuged as described for INTRALIPID above except that only one step centrifugation was used. Lipid-to-protein ratios of either 0.1, 0.3, or 0.6 ml of concentrated medium per ml INTRALIPID were used.

A sample from each top fraction was delipidated, fractionated by SDS-PAGE and the gels stained with Coomassie blue. ApoAI is the only major protein that is separated with the less dense INTRALIPID fraction, and increasing quantities of medium yield increasing amounts of purified apoAI. The purity of apoAI was about 95%, as determined by staining density on the gels. The fraction not treated with INTRALIPID contains some apoAI and other contaminating proteins secreted from the CHO cells.

The apoAI from the production run above was further purified for additional complexation studies as follows: The apoAI enriched, delipidated protein precipitate was dissolved in 0.01 M Tris-HCl, pH 8.3, buffer made in 6 M urea. A 50 µl aliquot was injected

in a high-performance liquid chromatography system (HPLC) with a C_{18} column equilibrated in a 20% acetonitrile, 0.1% trifluoroacetic acid (TFA) solution. After injection the sample was eluted with a gradient of acetonitrile from 20% to 70% containing 0.1% TFA at 2 ml/min flow rate to obtain a single major peak. The fractions corresponding to this peak were pooled and acetonitrile was removed by vacuum. The dried pellet was dissolved in phosphate buffer and analyzed by SDS-PAGE to obtain a single 25 kd band.

A 39-amino acid N-terminal segment of the purified protein was sequenced and 95% of the total proteins were shown to be mature protein. The remaining 5% contains the additional 6 amino acids of proapoAI sequence.

The purified apoAI was used to complex with repurified egg phosphatidylcholine (PC) obtained from Sigma Chemicals Co. (St. Louis, MO). The PC was dissolved in ethanol (4 mg/ml). The lipid solution (4 ml) was dried under vacuum to a thin film and hydrated with 2 ml of PBS, pH 7.4. The cloudy suspension was sonicated at 15°C for 1 hour under a stream of nitrogen. The sonicated suspension was centrifuged at 38 K rpm for 1 hour, and the supernatant which contains unilamellar and multilamellar liposomes was carefully removed.

The apoAI was incubated with the PC liposomes in a weight ratio of 1:5 at 37°C for 1 hour. After incubation, apoAI bound to liposomes was separated from free apoAI by gel filtration on 10% Agarose column. This material was then analyzed by negative-stain electron microscopy.

Figure 17 shows (a) the many disc-like structures which have formed in the presence of AI, and

(b) lack of identifiable lamellar vesicle structure. The disc-like structures are similar in appearance to nascent HDL-like particles isolated from liver perfusate (see Hamilton, R.L., et al, supra).

The ability of the purified apoAI to stabilize emulsions in serum was verified as follows. The emulsion was mixed with protein AI purified as above at a wt ratio of 100:2 mg emulsion lipid/mg AI. The mixture was incubated with shaking at 37°C for 1 hour, to bind apoAI to the emulsion. Unbound apoAI was removed by centrifugation.

Serum from rats treated with turpentine (5 ml/kg) or from control rats treated with saline were used. In each test, 180 µl of serum was incubated with 20 µl of lipid emulsion for 2 hours at 37°C with gentle shaking. After incubation, lipid-particle diameters were determined by laser light scattering using a sub-micron particle analyzer with optional size distribution processor analysis and multiple scattering angle detection (Coulter Model N4, Hialeah, FL).

The lipid emulsion without apoAI was unstable in serum from rats treated with turpentine and showed a bimodal distribution of sizes centered around 200 and 500 nm. In contrast, the emulsion containing apoAI showed no significant size change on exposure to serum obtained from turpentine-treated animals. Both emulsions were size stable in serum from control rats.

D.5.d. Atrial Naturetic Factor

CHO cells transformed with pMT'-ANF in a manner similar to that described above are verified to produce ANF when cultured in Harris F-12 medium supplemented with 10% fetal calf serum using radioimmunoassay and radiolabeling with ^{35}S methionine. In the

^{35}S-methionine assay, bands appear at 18 kd and 10 kd representing pro-ANF and a fragment thereof. An isolated colony showing this production, designated CHO-8/2-81 was deposited with ATCC on 9 April 1985 and has accession no. CRL-8782.

The ANF per se may be produced from the 18 kD pro-ANF by limited proteolysis using trypsin or kallikrein, as described in Currie et al (Proc Natl Acad Sci (USA) (1984) 81:1230-1233).

D.5.e. Erythropoietin

Plate Assays

Each of the plasmids pMT-Epo, pEpo-SV(10), pEpo'-SV(10) and pEpo-MT were used to transform CHO cells as described for the alternate constructions in the paragraphs above. Epo production levels were assessed by assaying the medium according to the method of Krystal, G., Exp Hematol (1983) 11:649-660, which employs tritiated thymidine uptake into spleen cells from phenylhydrazine-treated mice. (One mg of purified standard erythropoietin is believed to give 70,000 international units of activity.)

Pools containing pEpo-SV(10) transformants produced Epo at levels about ten-fold greater than those transformed with pMT-Epo under the same conditions though both were low producers: 8 IU/ml (0.1 µg/ml), as opposed to 0.5 IU/ml (0.01 µg/ml). Pools of cells transformed with the plasmid containing the altered gene, pEpo'-SV(10) produced 2080 IU/ml (30 µg/ml).

Cells transformed with pEpo-MT but not selected for cadmium resistance, produced Epo at the same level as those transformed with pMT-Epo; however when preselected for resistance at 5 µM Cd^{+} (as described for hGH in ¶D.5.a), a five-fold increase in expression

was obtained. In addition, Cd^{+2} resistance could be used to select for successful transformants in conjunction with pSV2-NEO. Cells which were treated with 2 µg or 10 µg, respectively, of pUC9-MT along with 15 µg pEpo-SV(10) and 1.5 µg pSV2-NEO were selectable for Cd^{+2}-resistant clones directly.

Production Runs

A clone which is particularly effective in producing erythropoietin, a pEpo'-SV10 transformant designated Epo-E, was chosen by screening the Epo'-SV10 pool in 96 well plates using immunoblot, picking the 12 best clones, transferring these clones into a 12 well plate, inducing for erythropoietin production, assaying by immunoblot and picking one of four clones which produce approximately 10,000 units/ml for roller bottle growth and induction. This clone, designated Epo-E, is used to seed fifteen 1750 cm^2 roller bottles containing 500 ml medium each at 1/10 confluence. The cells were grown to confluence in 4 days, washed twice with PBS, and fed serum free medium containing 60 µM zinc and 30 µM Fe^{+2} to induce production of the desired protein. Four days post induction, the cells maintained the 10,000 unit/ml production level, resulting in the production of 75 million units of erythropoietin for the 7.5 l run.

D.5.f. Renin

Plate Assay

Pools of CHO cells selected for G418 resistance after transformation with pMT-PPRen produce renin after induction with 5 x 10^{-5} zinc sulfate and 10^{-6} M dexamethasone at levels of 0.02-0.2 µg/ml. The renin so produced was shown to be glycosylated by comparison

of SDS gels run on ^{35}S methionine-labeled secreted renin with and without treatment with endo F.

By appropriate selection, much higher levels of renin production can be achieved. In one approach, cells from the transformant pool were plated out in the presence of 5×10^{-5} M zinc sulfate in serum-free medium, and colonies which grew under these conditions were picked, expanded in serum-containing medium, and then assayed for renin production under standard induction conditions as described above. The highest renin-producing clones obtained after this process produced an average of 0.8 µg/ml, and one clone of this class, designated CBI-2B5 (supra) was deposited at ATCC with accession no. CRL 8758.

In a second approach, the cells from the pool were plated and replicated onto a polyester sheet. The replica sheet was then placed against a nitrocellulose filter for binding of the secreted cell protein. The filter was then reacted with with antiprorenin antiserum followed by treating with ^{125}I protein A. Clones which appeared to have the highest levels of renin secretion according to this assay were picked, expanded in serum-containing medium, and reassayed for activity under standard conditions. Clones were obtained producing 1-4 µg/ml of renin, and only one such clone, arbitrarily chosen, CBI-AA2, was selected for study under production conditions, as described below.

Production Runs

The CHO transformant cells designated CBI-AA2 were prepared for roller bottle culture growth in 100 mm tissue culture dishes containing 10 ml complete media (10% FCS in F12/DMEM21 and containing 50 U/ml penicillin, 50 µg/ml streptomycin, or 400 µg/ml

G-418. The cells are grown until attached (30 min), the media withdrawn, and cells refed with fresh complete media. The cells were allowed to grow to confluence, and passaged by trypsin using standard protocols for expansion. 850-cm square roller bottles were seeded with cells from one confluent flask (4×10^7 cells) in 200 ml complete medium supplemented with 10 mM Hepes, pH 7.2. The cells were grown at 37°C with rotation until confluent.

To induce production of renin in serum-free medium, the complete media were aspirated from the roller bottles and the cells were washed with 200 ml DMEM21. The cells were then fed with approximately 100-200 ml serum-free induction medium (SFIM). SFIM consists of F12/DMEM21 (1:1), containing 1 mM Hepes, 3×10^{-5} M $FeSO_4$, 5×10^{-5} M $ZnSO_4$, and 10^{-6} M dexamethasone. The cells are refed with SFIM on a cycle determined by the amount of secreted protein -- i.e., when approximately 100 µg/ml total protein has been secreted, as determined by Bradford assay. In general this level of total protein production occurs in approximately 2 days. CBI-AA2 showed renin production levels of 1 µg/ml or approximately 0.5 µg/ml/day.

AtT-20 Transformants

AtT-20 cells were grown as described for CHO cells above in preparation for transformation, except that a 15% CO_2 atmosphere rather than a 5% CO_2 atmosphere was used. Transformation and selection for successful transformants by G418 resistance was as described for CHO cells, and the selected cells were screened for renin production in the supernatant fluid using the plate assays described for the CHO cells

producing renin above, except that only serum-containing media were used.

High producing lines secreted both renin and prorenin into the medium. A comparison of the secreted material precipitable with human renin antiserum is shown in Figure 18. Figure 18 shows an SDS gel of immunoprecipitated proteins from supernatants of ^{35}S-methionine labeled cells: untransfected AtT-20 cells, AtT-20 cells transfected with pMT-PPRen and CHO cells transformed with this plasmid. The cells were labeled for 15 hr with ^{35}S-methionine after induction as described above. The medium was collected, and the supernatant immunoprecipitated and subjected to SDS-PAGE.

Lane 1 represents the immunoprecipitates from these initial supernatants from untransformed cells, lane 2 transformed AtT-20 cells, and lane 3 transformed CHO cells. As shown, the transfected AtT-20 cells secrete both prorenin and renin; transfected CHO cells secrete only the prorenin form.

Cultures containing successful transformants are subjected to selection for cells capable of being maintained under serum free conditions. The cultures are grown in 10% FCS to 1/2 confluence, and then switched to serum-free medium. The culture plates are fed with serum-free medium until most but not all cells have died, whereon medium containing 10% FCS is added to allow division of surviving cells. The surviving cells are passaged, replated, and after 1 day on 10% FCS are again switched to serum-free medium. Cells which survive 2 weeks are expanded by addition of medium containing 10% FCS, cloned at limiting dilution, and assayed for renin production after induction using zinc ion and iron as induction mediator as described above.

Claims

1. A regulatable expression system for a desired coding sequence which comprises mammalian host cells which have been transformed with a DNA sequence comprising the metallothionein II (MT-II) promoter operably linked to said desired coding sequence, wherein said mammalian host cells are maintained on a serum-free medium and have been induced with an effective amount of an induction mediator and an effective amount of non-toxic metal ions.

2. The system of claim 1 wherein the induction mediator is >5 μg/ml human transferrin or 1-3 x 10^{-5} M iron ion and wherein the non-toxic metal ions are zinc ions in a concentration range of 2 x 10^{-5} to 2 x 10^{-4} M.

3. The system of claim 1 wherein the DNA sequence further includes an enhancer operably linked to the promoter.

4. The system of claim 1, 2 or 3 wherein the cells have been cotransformed with an amplifiable toxin-resistance conferring gene.

5. The system of claim 4 wherein the toxin-resistance conferring gene is the MT gene.

6. The system of claims 1-5 wherein the coding sequence encodes a protein selected from the group consisting of alveolar surfactant protein (ASP), human growth hormone (hGH), apolipoproteins AI and AII (apoAI

and apoAII), atrial natriuretic factor (ANF), erythropoietin (Epo), and renin.

7. A method for obtaining expression of a desired coding sequence in mammalian cells which comprises inducing the cells of claims 1-6.

8. A protein produced by the method of claim 7.

9. The protein of claim 8 which is selected from the group consisting of alveolar surfactant protein (ASP), human growth hormone (hGH), apolipoproteins AI and AII (apoAI and apoAII), atrial natriuretic factor (ANF), erythropoietin (Epo), and renin.

10. The hGH of claim 9 which comprises about 10% by weight of approximately 20 kD protein and about 90% by weight of approximately 22 kD protein.

11. An expression system for enhanced production of a desired protein which system comprises mammalian host cells transformed with a first DNA sequence comprising the coding sequence for said protein operably linked to a promoter and cotransformed with a second DNA sequence capable of expressing the MT protein.

12. The expression system of claim 11 wherein the transformed cells have been selected for cadmium ion resistance, and wherein the first DNA sequence comprises the MT-II promoter operably linked to the coding sequence.

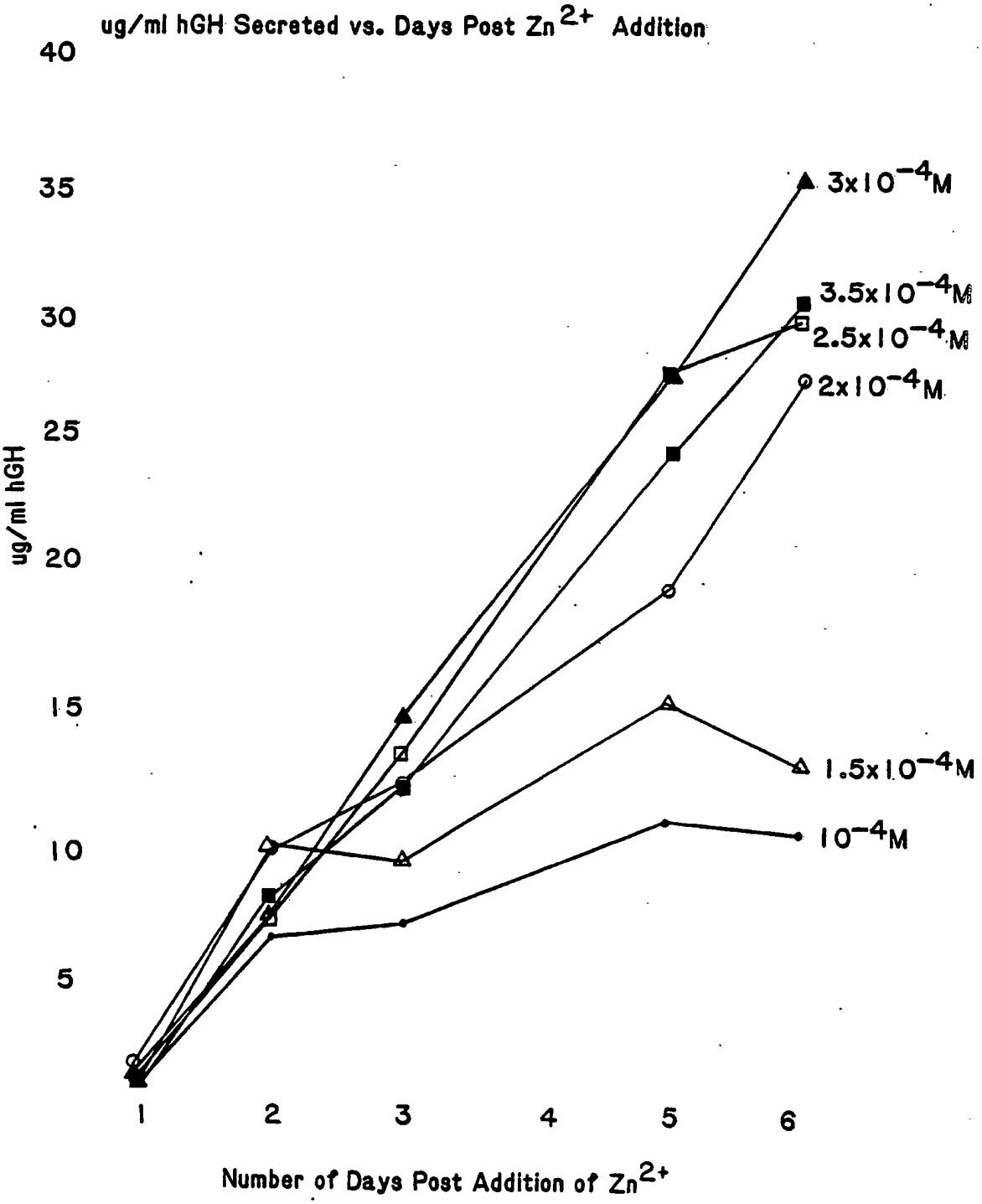
13. The expression system of claim 11 or 12 wherein the cells are maintained on serum free medium

and induced with an effective amount of non-toxic metal ion.

14. The expression system of claim 13 wherein the non-toxic metal ions are zinc ions in a concentration range of 2×10^{-5} to 2×10^{-4} M.

15. A recombinantly produced preparation of hGH which comprises about 10% by weight of approximately 20 kD protein and about 90% by weight of approximately 22 kD protein.

16. A recombinantly produced preparation of hASP which comprises about 70% by weight of protein with Glu at the N-terminus and about 30% by weight of protein with Val at the N-terminus.


ug/ml hGH Secreted vs. Days Post Zn^{2+} Addition
ug/ml hGH
40
35
30
25
20
15
10
5
1
2
3
4
5
6
$3x10^{-4}M$
$3.5x10^{-4}M$
$2.5x10^{-4}M$
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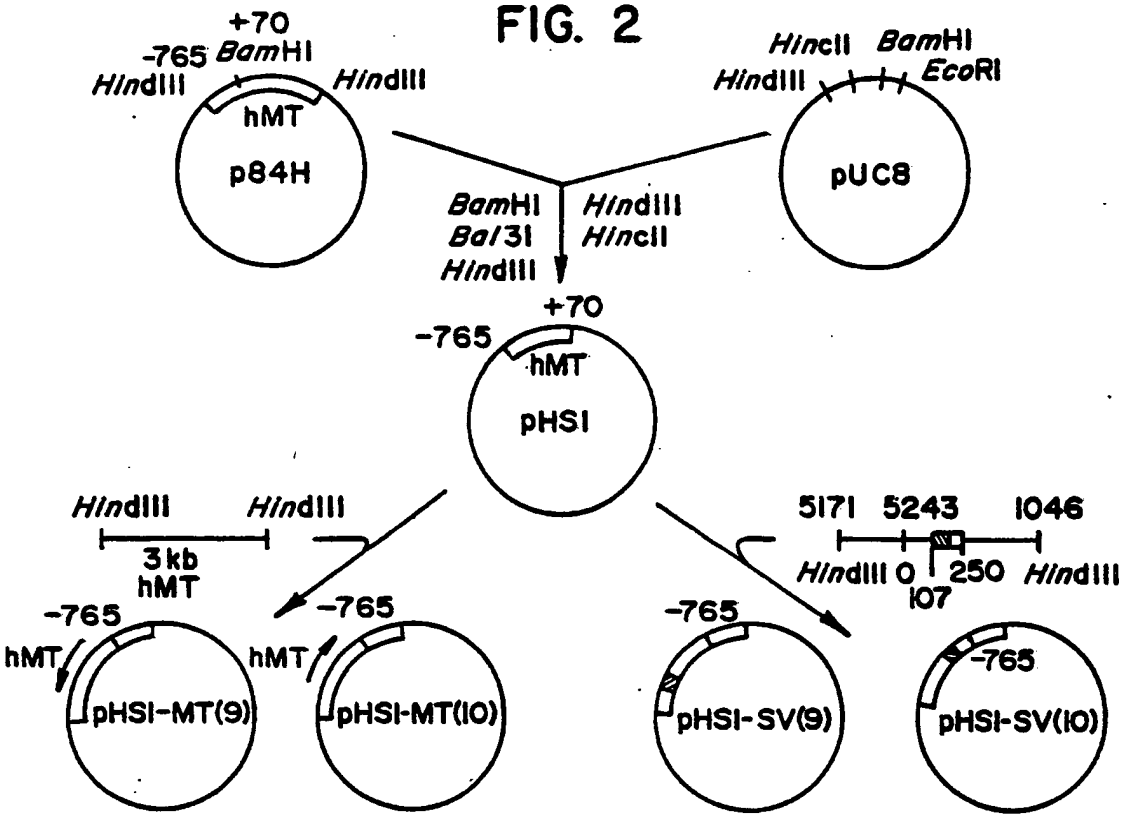
$10^{-4}M$
Number of Days Post Addition of Zn^{2+}

FIG. 1

FIG. 2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HindIII
hMT
p84H
HincII
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Bal31
HindIII
HindIII
HincII
+70
-765
hMT
pHSI
HindIII
HindIII
3kb
hMT
-765
hMT
pHSI-MT(9)
-765
hMT
pHSI-MT(10)
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HindIII
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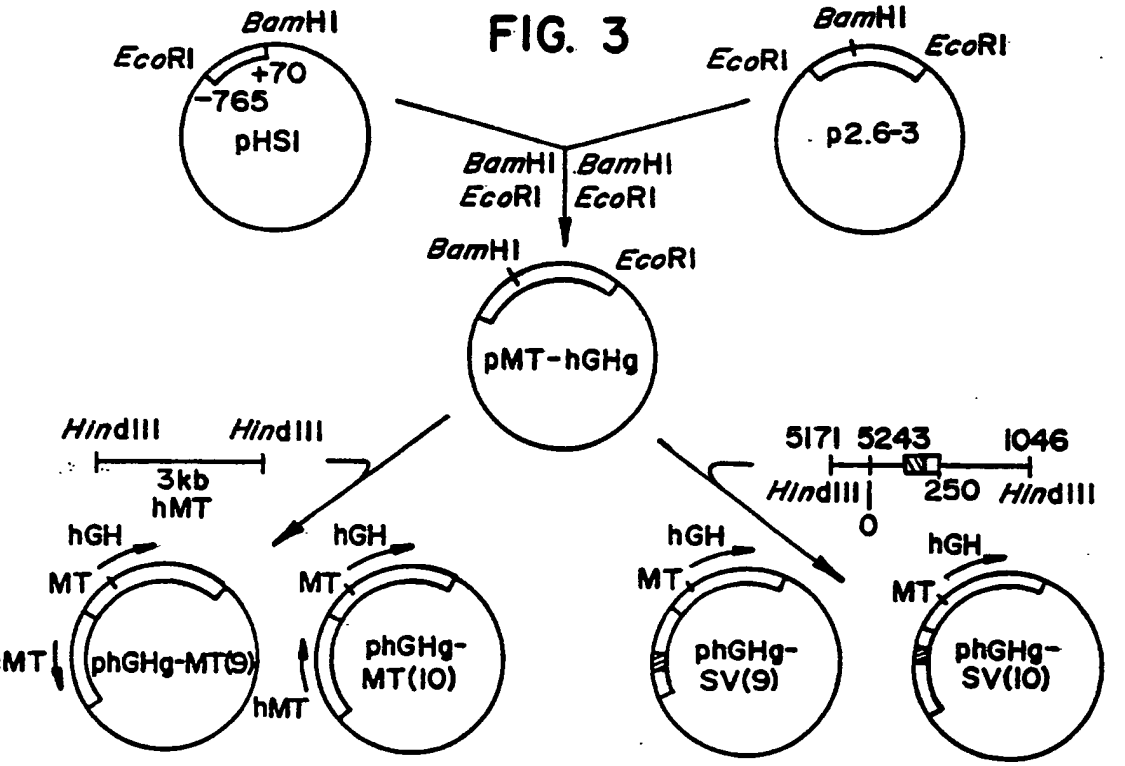
-765
pHSI-SV(9)
-765
pHSI-SV(10)

FIG. 3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hGH
MT
hMT
phGHg-MT(9)
hGH
MT
hMT
phGHg-MT(10)
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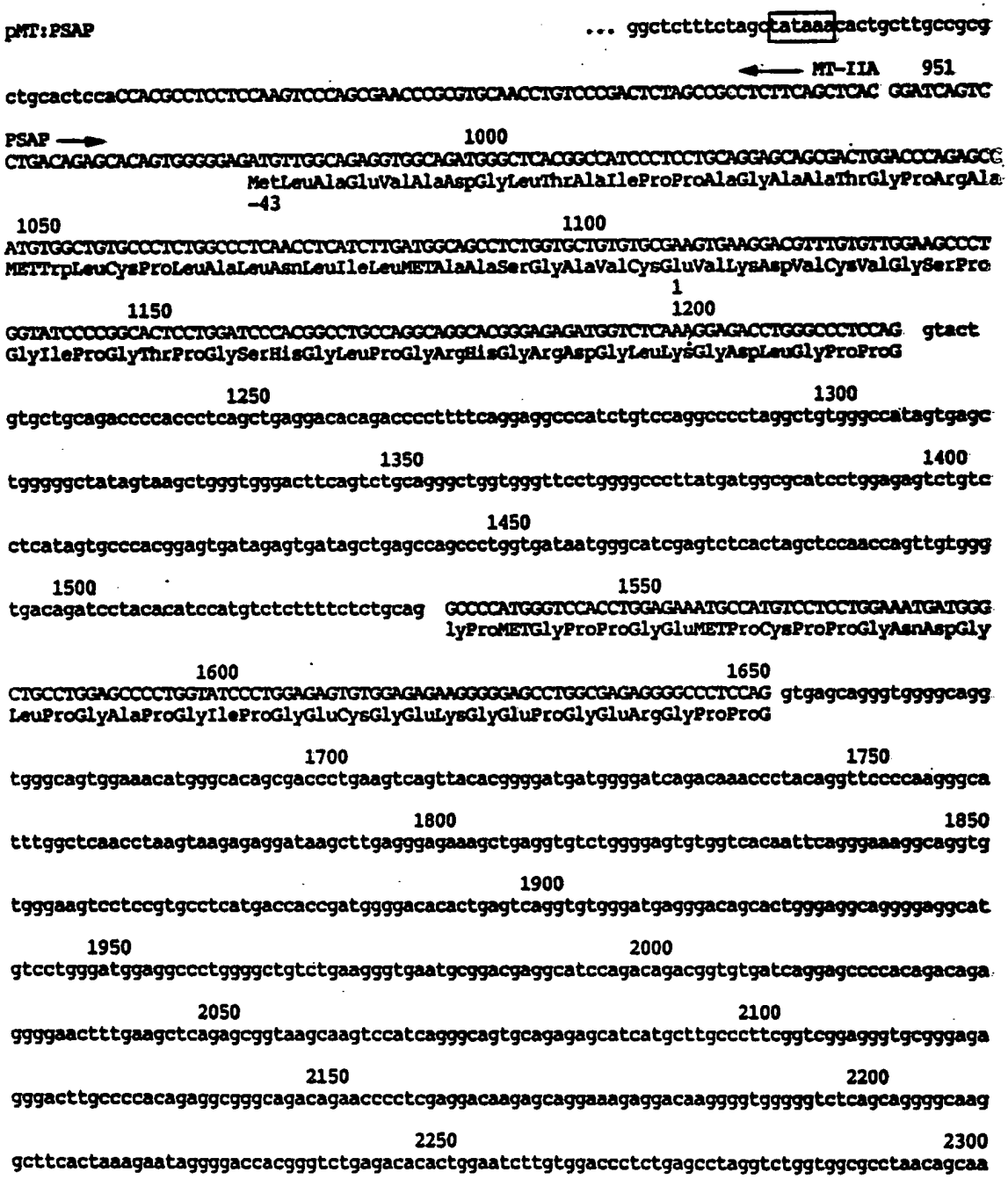
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1
1150
1200
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1300
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1650
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FIG. 4-1

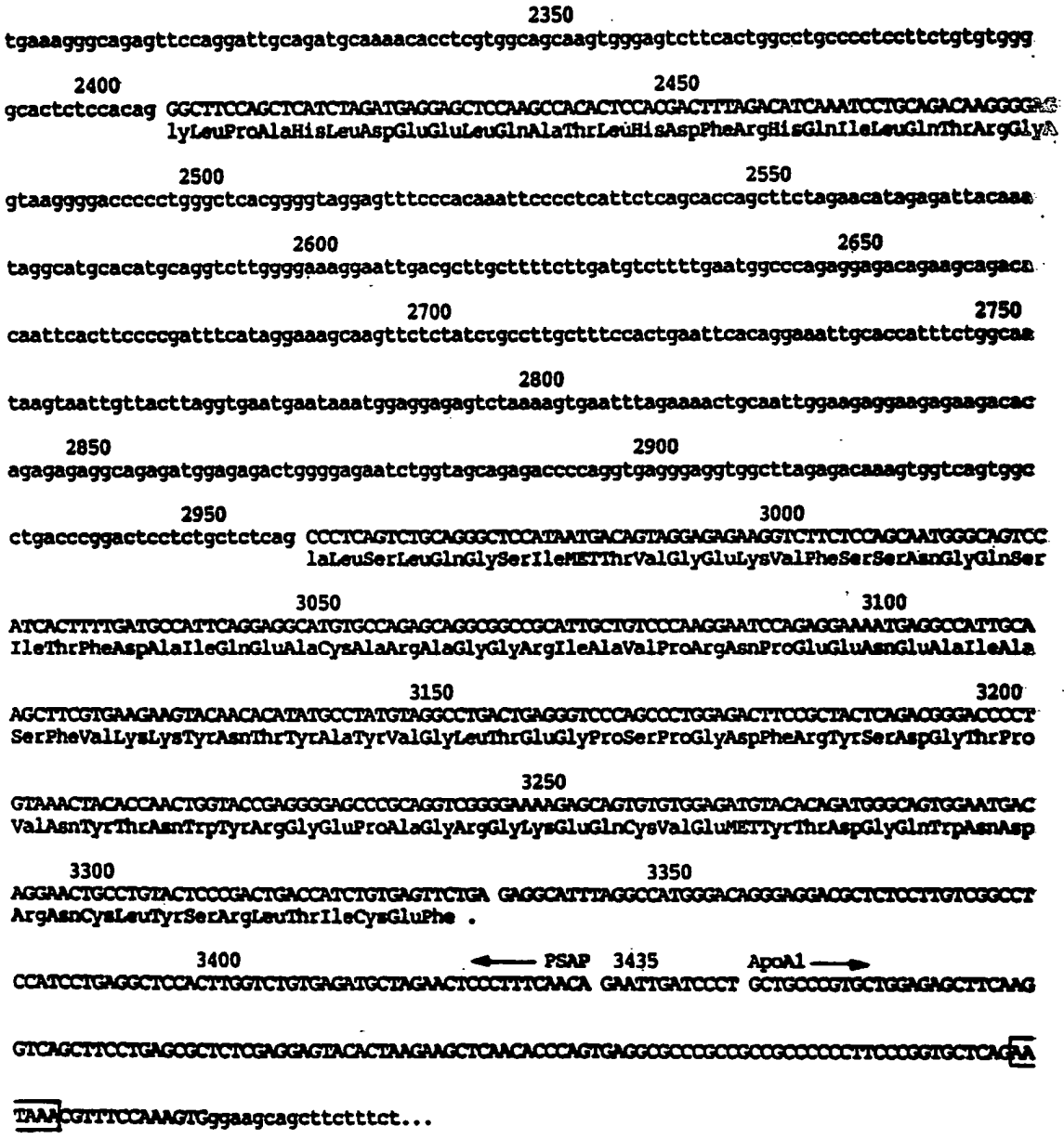


FIG. 4-2 pMT-Apo:gHS (HinfI/EcoRI)

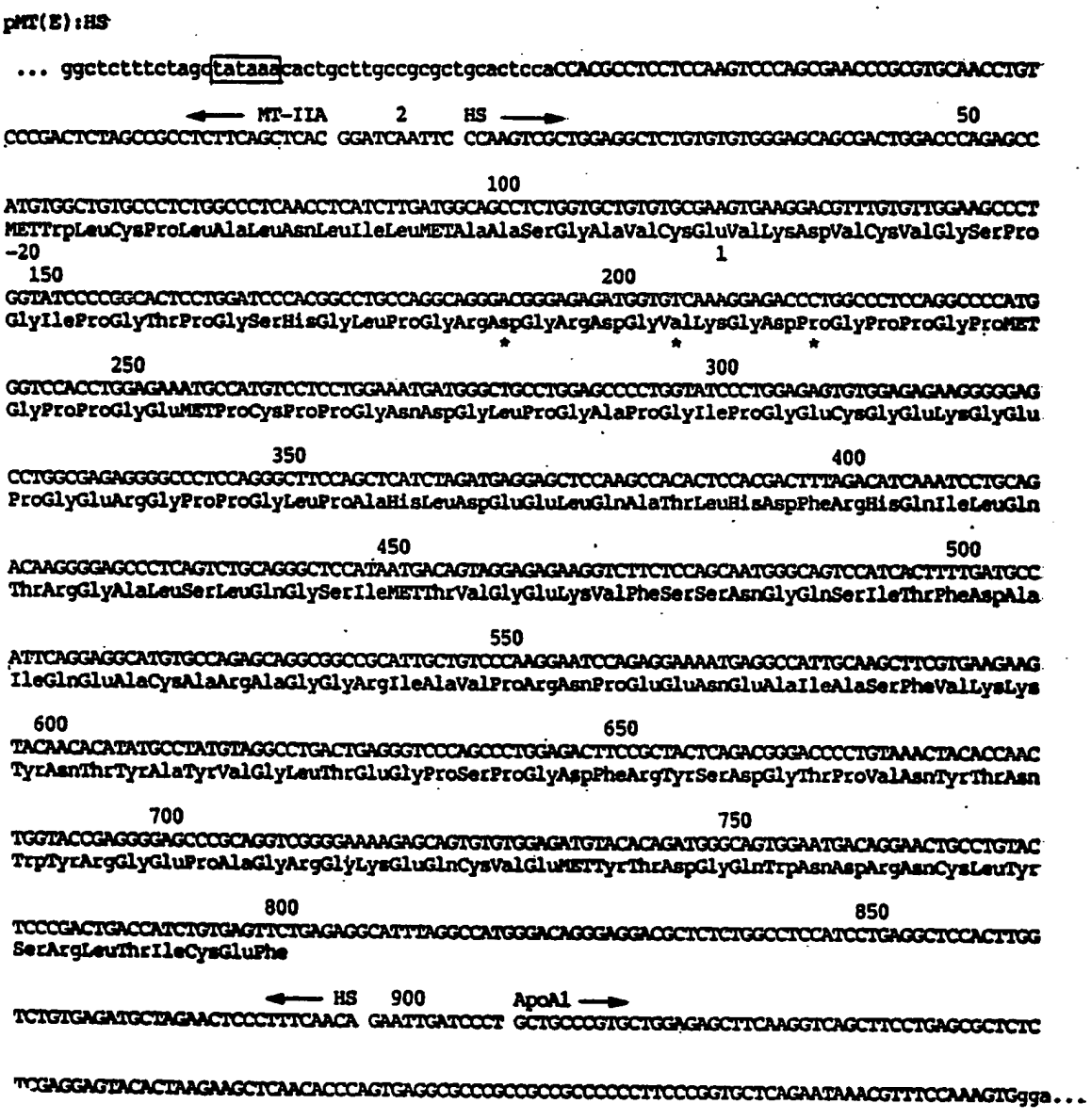


FIG. 5 pASPc – SV(10)

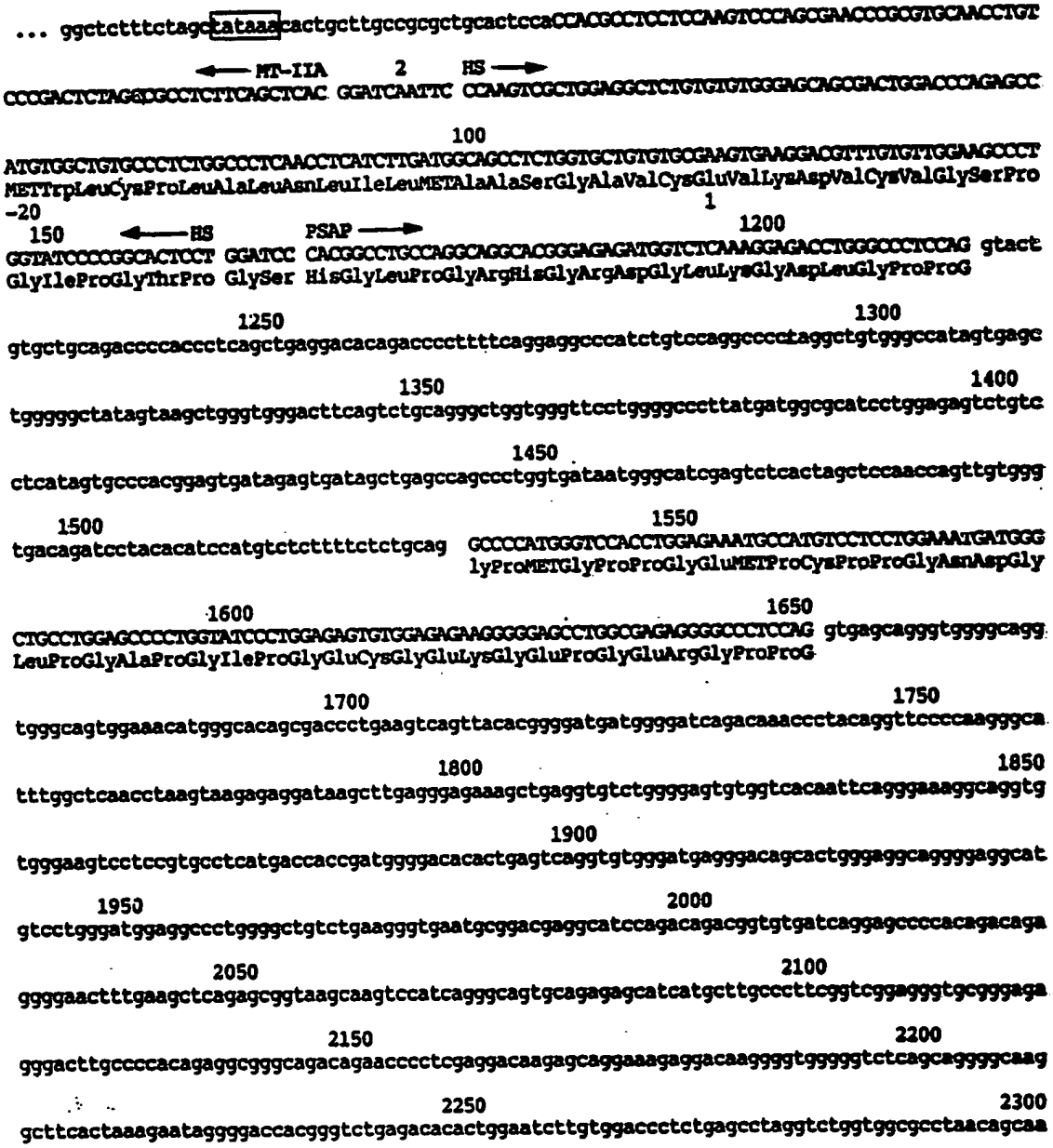

... ggctctttctagctataaacactgcttgccgcgctgcactccaCCACGCCTCCTCCAAGTCCCAGCGAACCCGCGTGCAACCTGT
MT-IIA 2 HS
CCCGACTCTAGAGGCCTCTTCAGCTCAC GGATCAATTC CCAAGTCGCTGGAGGCTCTGTGTGTGGGAGCAGCGACTGGACCCAGAGCC
100
ATGTGGCTGTGCCCTCTGGCCCTCAACCTCATCTTGATGGCAGCCTCTGGTGCTGTGTGCGAAGTGAAGGACGTTTGTGTTGGAAGCCCT
METTrpLeuCysProLeuAlaLeuAsnLeuIleLeuMETAlaAlaSerGlyAlaValCysGluValLysAspValCysValGlySerPro
-20 1
150 HS PSAP 1200
GGTATCCCCGGCACTCCT GGATCC CACGGCCTGCCAGGCAGGCACGGGAGAGATGGTCTCAAAGGAGACCTGGGCCCTCCAG gtact
GlyIleProGlyThrPro GlySer HisGlyLeuProGlyArgHisGlyArgAspGlyLeuLysGlyAspLeuGlyProProG
1250 1300
gtgctgcagaccccaccctcagctgaggacacagacccctttcaggaggcccatctgtccaggcccctaggctgtgggccatagtgagc
1350 1400
tgggggctatagtaagctgggtgggacttcagtctgcagggctggtgggttcctggggcccttatgatggcgcatcctggagagtctgtc
1450
ctcatagtgcccacggagtgatagagtgatagctgagccagccctggtgataatgggcatcgagtctcactagctccaaccagttgtggg
1500 1550
tgacagatcctacacatccatgtctcttttctctgcag GCCCCATGGGTCCACCTGGAGAAATGCCATGTCCTCCTGGAAATGATGGG
lyProMETGlyProProGlyGluMETProCysProProGlyAsnAspGly
1600 1650
CTGCCTGGAGCCCCTGGTATCCCTGGAGAGTGTGGAGAGAAGGGGGAGCCTGGCGAGAGGGGCCCTCCAG gtgagcagggtggggcagg
LeuProGlyAlaProGlyIleProGlyGluCysGlyGluLysGlyGluProGlyGluArgGlyProProG
1700 1750
tgggcagtggaaacatgggcacagcgaccctgaagtcagttacacggggatgatggggatcagacaaaccctacaggttccccaagggca
1800 1850
tttggctcaacctaagtaagagaggataagcttgagggagaaagctgaggtgtctggggagtgtggtcacaattcagggaaaggcaggtg
1900
tgggaagtcctccgtgcctcatgaccaccgatggggacacactgagtcaggtgtgggatgagggacagcactgggaggcaggggaggcat
1950 2000
gtcctgggatggaggccctggggctgtctgaagggtgaatgcggacgaggcatccagacagacggtgtgatcaggagccccacagacaga
2050 2100
ggggaactttgaagctcagagcggtaagcaagtccatcagggcagtgcagagagcatcatgcttgcccttcggtcggagggtgcgggaga
2150 2200
gggacttgccccacagaggcgggcagacagaaccctcgaggacaagagcaggaaagaggacaagggtgggggtctcagcaggggcaag
2250 2300
gcttcactaaagaataggggaccacgggtctgagacacactggaatcttgtggaccctctgagcctaggtctggtggcgcctaacagcaa

FIG. 6-1

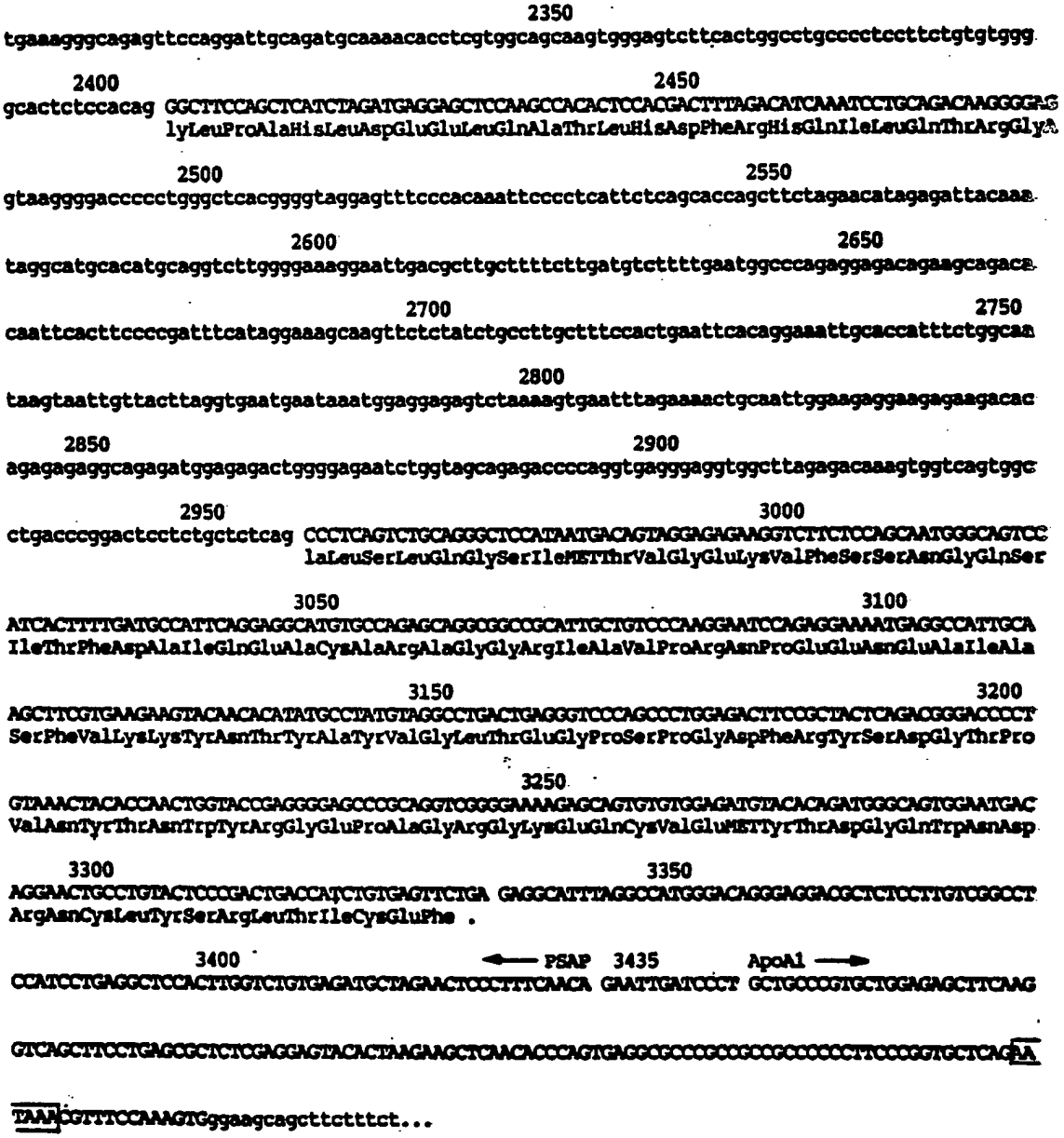


FIG. 6-2 pASPcg – SV(10)

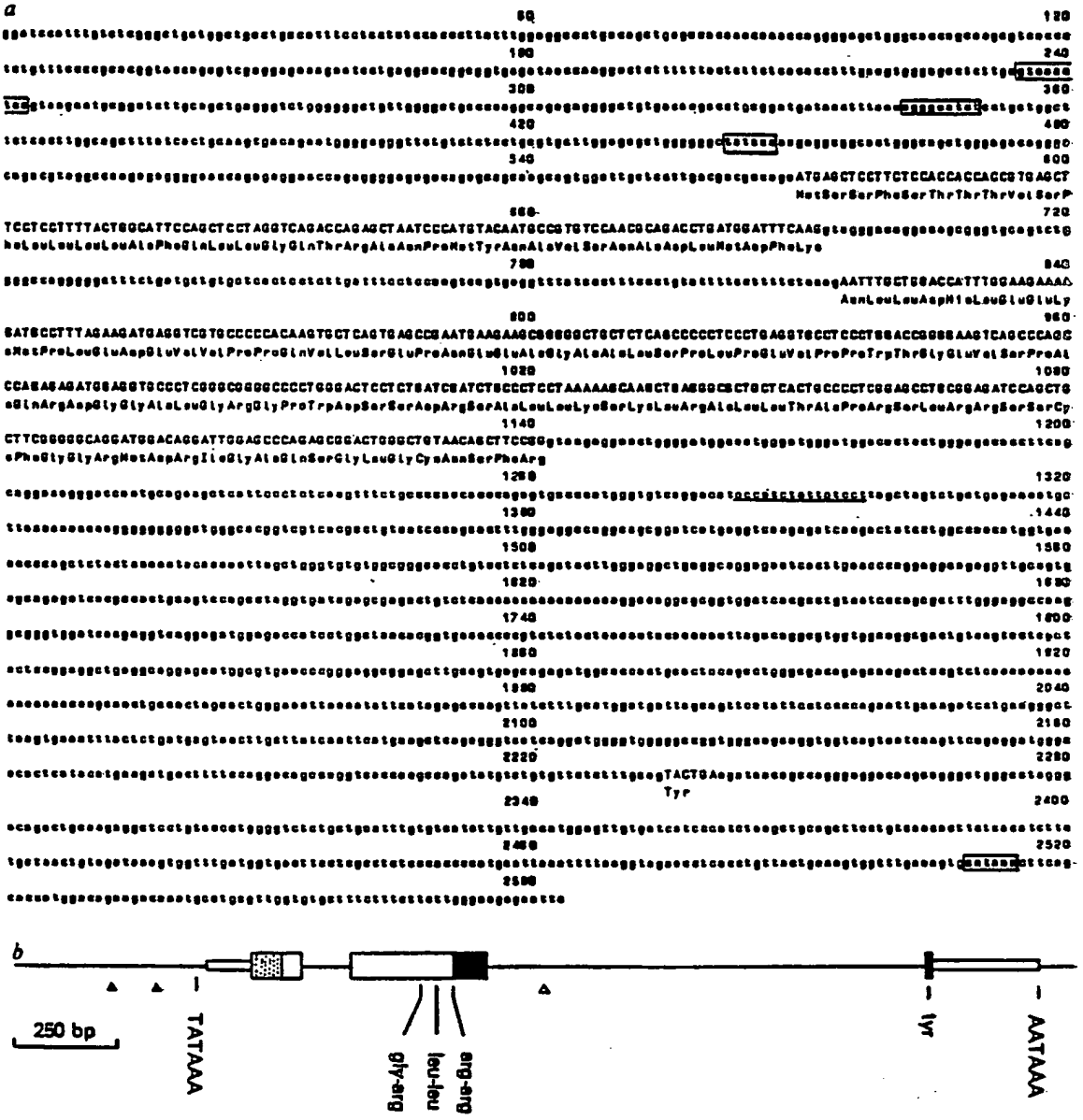


FIG. 7

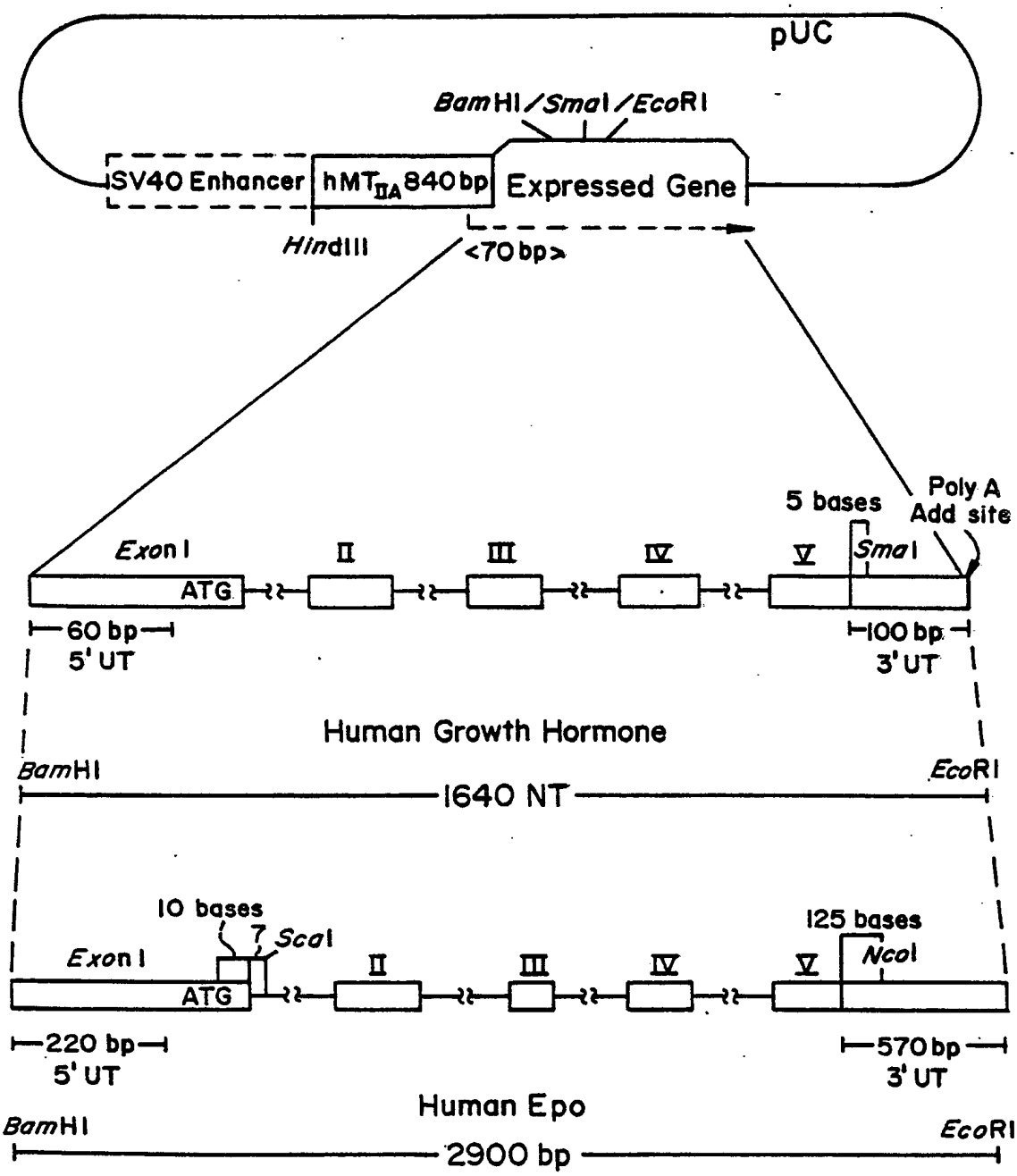

pUC
BamHI/SmaI/EcoRI
SV40 Enhancer
hMT$_{IIA}$ 840 bp
Expressed Gene
HindIII
<70 bp>
5 bases
Poly A
Add site
Exon I
II
III
IV
V
SmaI
ATG
60 bp
5' UT
100 bp
3' UT
Human Growth Hormone
BamHI
EcoRI
1640 NT
10 bases
7
ScaI
125 bases
NcoI
Exon I
ATG
220 bp
5' UT
570 bp
3' UT
Human Epo
BamHI
EcoRI
2900 bp

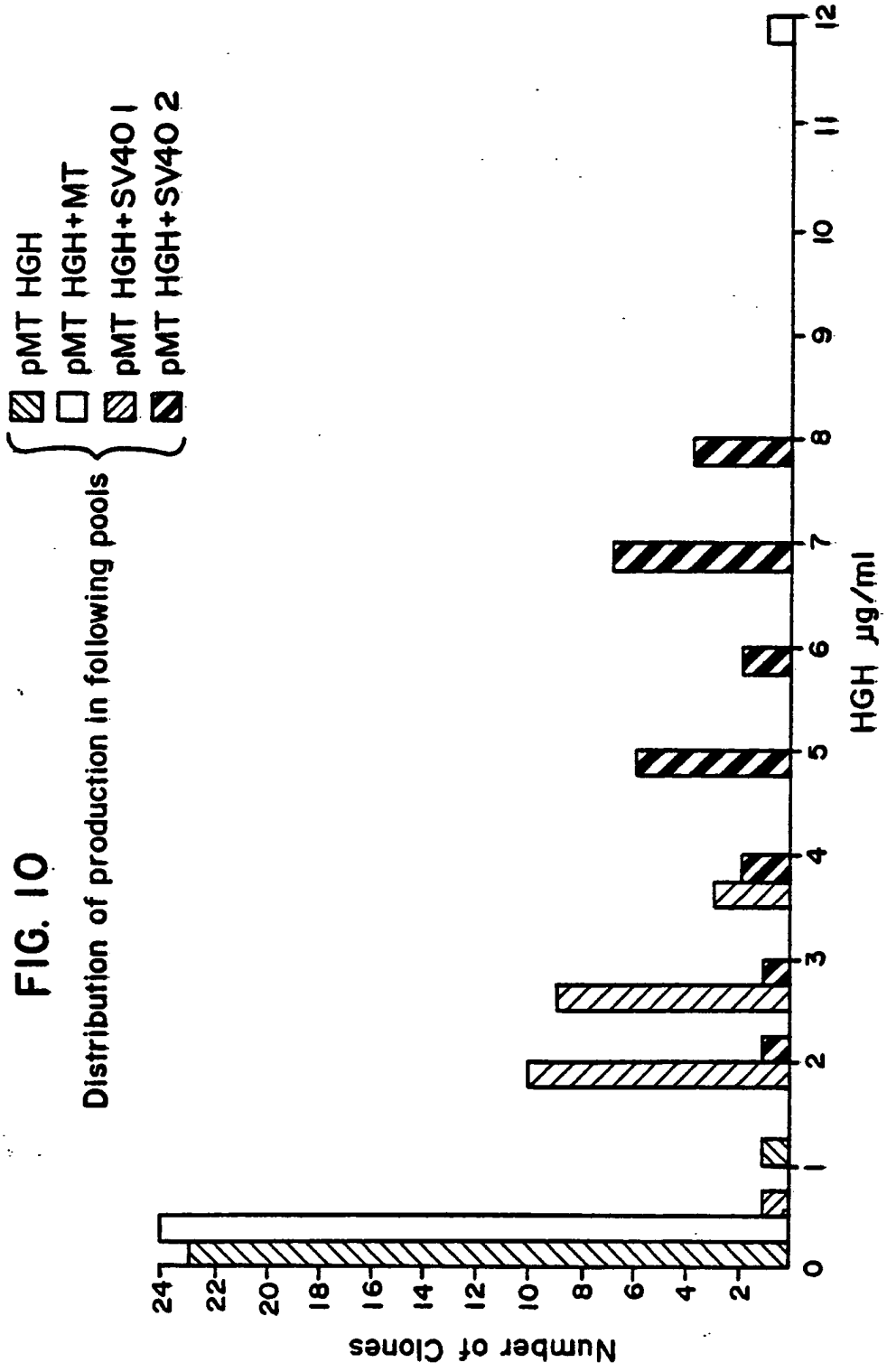
FIG. 8

```
 -42 AACCTCAGTGGATCTCAGAGAGAGCCCCAGACTGAGGGAAGC   -1

   1 ATG GAT GGA TGG AGA AGG ATG CCT CGC TGG GGA CTG CTG CTG CTG CTC TGG GGC TCC TGT   60
     Met Asp Gly Trp Arg Arg Met Pro Arg Trp Gly Leu Leu Leu Leu Leu Trp Gly Ser Cys
       1                  (-60)          10                          (-50)        20
  61 ACC TTT GGT CTC CCG ACA GAC ACC ACC ACC TTT AAA CGG ATC TTC CTC AAG AGA ATG CCC  120
     Thr Phe Gly Leu Pro Thr Asp Thr Thr Thr Phe Lys Arg Ile Phe Leu Lys Arg Met Pro
                            (-40)        30                          (-30)        40
 121 TCA ATC CGA GAA AGC CTG AAG GAA CGA GGT GTG GAC ATG GCC AGG CTT GGT CCC GAG TGG  180
     Ser Ile Arg Glu Ser Leu Lys Glu Arg Gly Val Asp Met Ala Arg Leu Gly Pro Glu Trp
                            (-20)        50                          (-10)        60
 181 AGC CAA CCC ATG AAG AGG CTG ACA CTT GGC AAC ACC ACC TCC TCC GTG ATC CTC ACC AAC  240
     Ser Gln Pro Met Lys Arg Leu Thr Leu Gly Asn Thr Thr Ser Ser Val Ile Leu Thr Asn
                        (-1)(1)          70                      (10)             80
 241 TAC ATG GAC ACC CAG TAC TAT GGC GAG ATT GGC ATC GGC ACC CCA CCC CAG ACC TTC AAA  300
     Tyr Met Asp Thr Gln Tyr Tyr Gly Glu Ile Gly Ile Gly Thr Pro Pro Gln Thr Phe Lys
                        (20)             90                      (30)            100
 301 GTC GTC TTT GAC ACT GGT TCG TCC AAT GTT TGG GTG CCC TCC TCC AAG TGC AGC CGT CTC  360
     Val Val Phe Asp Thr Gly Ser Ser Asn Val Trp Val Pro Ser Ser Lys Cys Ser Arg Leu
                        (40)            110                      (50)            120
 361 TAC ACT GCC TGT GTG TAT CAC AAG CTC TTC GAT GCT TCG GAT TCC TCC AGC TAC AAG CAC  420
     Tyr Thr Ala Cys Val Tyr His Lys Leu Phe Asp Ala Ser Asp Ser Ser Ser Tyr Lys His
                        (60)            130                      (70)            140
 421 AAT GGA ACA GAA CTC ACC CTC CGC TAT TCA ACA GGG ACA GTC AGT GGC TTT CTC AGC CAG  480
     Asn Gly Thr Glu Leu Thr Leu Arg Tyr Ser Thr Gly Thr Val Ser Gly Phe Leu Ser Gln
                        (80)            150                      (90)            160
 481 GAC ATC ATC ACC GTG GGT GGA ATC ACG GTG ACA CAG ATG TTT GGA GAG GTC ACG GAG ATG  540
     Asp Ile Ile Thr Val Gly Gly Ile Thr Val Thr Gln Met Phe Gly Glu Val Thr Glu Met
                       (100)            170                     (110)            180
 541 CCC GCC TTA CCC TTC ATG CTG GCC GAG TTT GAT GGG GTT GTG GGC ATG GGC TTC ATT GAA  600
     Pro Ala Leu Pro Phe Met Leu Ala Glu Phe Asp Gly Val Val Gly Met Gly Phe Ile Glu
                       (120)            190                     (130)            200
 601 CAG GCC ATT GGC AGG GTC ACC CCT ATC TTC GAC AAC ATC ATC TCC CAA GGG GTG CTA AAA  660
     Gln Ala Ile Gly Arg Val Thr Pro Ile Phe Asp Asn Ile Ile Ser Gln Gly Val Leu Lys
                       (140)            210                     (150)            220
 661 GAG GAC GTC TTC TCT TTC TAC TAC AAC AGA GAT TCC GAG AAT TCC CAA TCG CTG GGA GGA  720
     Glu Asp Val Phe Ser Phe Tyr Tyr Asn Arg Asp Ser Glu Asn Ser Gln Ser Leu Gly Gly
                       (160)            230                     (170)            240
 721 CAG ATT GTG CTG GGA GGC AGC GAC CCC CAG CAT TAC GAA GGG AAT TTC CAC TAT ATC AAC  780
     Gln Ile Val Leu Gly Gly Ser Asp Pro Gln His Tyr Glu Gly Asn Phe His Tyr Ile Asn
                       (180)            250                     (190)            260
 781 CTC ATC AAG ACT GGT GTC TGG CAG ATT CAA ATG AAG GGG GTG TCT GTG GGG TCA TCC ACC  840
     Leu Ile Lys Thr Gly Val Trp Gln Ile Gln Met Lys Gly Val Ser Val Gly Ser Ser Thr
                       (200)            270                     (210)            280
 841 TTG CTC TGT GAA GAC GGC TGC CTG GCA TTG GTA GAC ACC GGT GCA TCC TAC ATC TCA GGT  900
     Leu Leu Cys Glu Asp Gly Cys Leu Ala Leu Val Asp Thr Gly Ala Ser Tyr Ile Ser Gly
                       (220)            290                     (230)            300
 901 TCT ACC AGC TCC ATA GAG AAG CTC ATG GAG GCC TTG GGA GCC AAG AAG AGG CTG TTT GAT  960
     Ser Thr Ser Ser Ile Glu Lys Leu Met Glu Ala Leu Gly Ala Lys Lys Arg Leu Phe Asp
                       (240)            310                     (250)            320
 961 TAT GTC GTG AAG TGT AAC GAG GGC CCT ACA CTC CCC GAC ATC TCT TTC CAC CTG GGA GGC 1020
     Tyr Val Val Lys Cys Asn Glu Gly Pro Thr Leu Pro Asp Ile Ser Phe His Leu Gly Gly
                       (260)            330                     (270)            340
1021 AAA GAA TAC ACG CTC ACC AGC GCG GAC TAT GTA TTT CAG GAA TCC TAC AGT AGT AAA AAG 1080
     Lys Glu Tyr Thr Leu Thr Ser Ala Asp Tyr Val Phe Gln Glu Ser Tyr Ser Ser Lys Lys
                       (280)            350                     (290)            360
1081 CTG TGC ACA CTG GCC ATC CAC GCC ATG GAT ATC CCG CCA CCC ACT GGA CCC ACC TGG GCC 1140
     Leu Cys Thr Leu Ala Ile His Ala Met Asp Ile Pro Pro Pro Thr Gly Pro Thr Trp Ala
                       (300)            370                     (310)            380
1141 CTG GGG GCC ACC TTC ATC CGA AAG TTC TAC ACA GAG TTT GAT CGC CGT AAC AAC CGC ATT 1200
     Leu Gly Ala Thr Phe Ile Arg Lys Phe Tyr Thr Glu Phe Asp Arg Arg Asn Asn Arg Ile
                       (320)            390                     (330)            400
1201 GGC TTC GCC TTG GCC CGC TGAGGCCCTCTGCCACCCAGGCAGGCCCTGCCTTCAGCCCTGGCCCAGAGCTGGA 1273
     Gly Phe Ala Leu Ala Arg
                       (340)406
1274 ACACTCTCTGAGATGCCCCTCTGCCTGGGCTTATGCCCTCAGATGGAGACATTGGATGTCGAGCTCCTGCTGGATGCGT 1352

1353 GCCCTGACCCCTGCACCAGCCCTTCCCTGCTTTGAGGACAAAGAGAATAAAGACTTCATGTTCAC
```

FIG. 9

SUBSTITUTE SHEET

FIG. 10


Distribution of production in following pools
pMT HGH
pMT HGH+MT
pMT HGH+SV40 1
pMT HGH+SV40 2
Number of Clones
24
22
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
11
12
HGH µg/ml

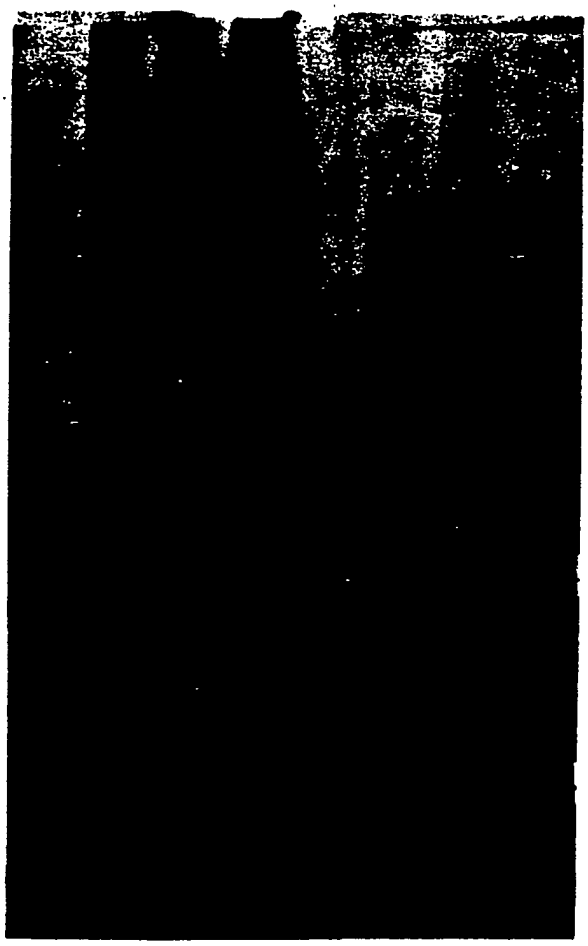

-25.7K
-18.4K
-12.3K
-6K
-3K
total secreted protein
anti-hGH ppt.
1 - CBI 37
2 - CBI 25
3 - CHO:K1
4 - CBI 37
5 - CBI 25
6 - CHO:K1
low M.W. standards

FIG. 11

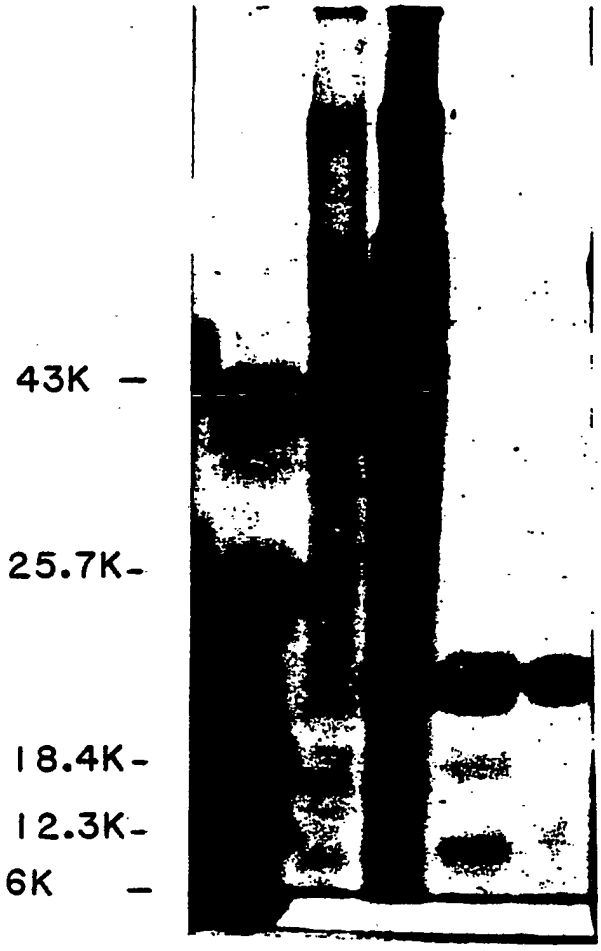

43K
25.7K
18.4K
12.3K
6K
total secreted protein
low M.W. standards
CHO:K1
CBI 37
CBI 37 after Gel permeation
NPa hGH
1
2
3
4
5

FIG. 12

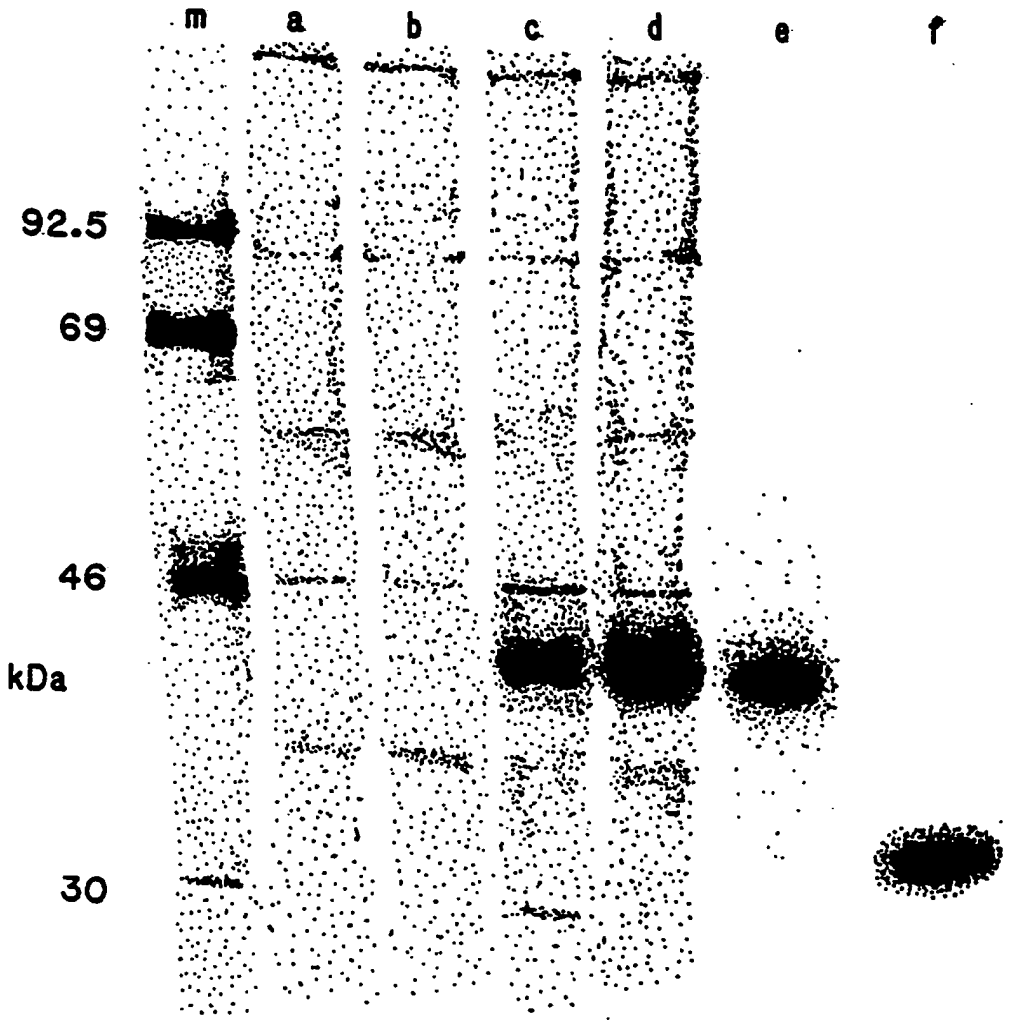


FIG. 13

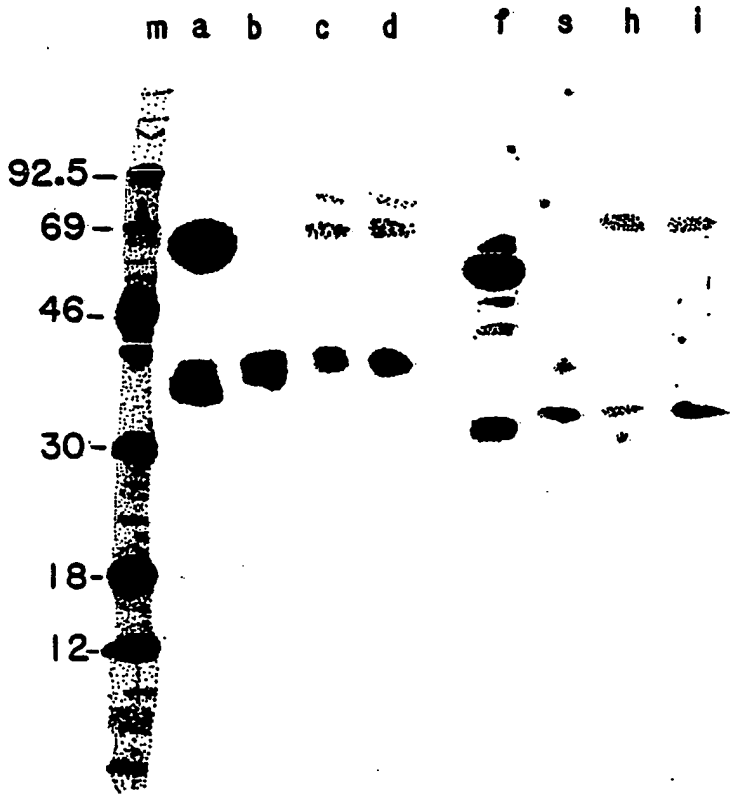


FIG. 14

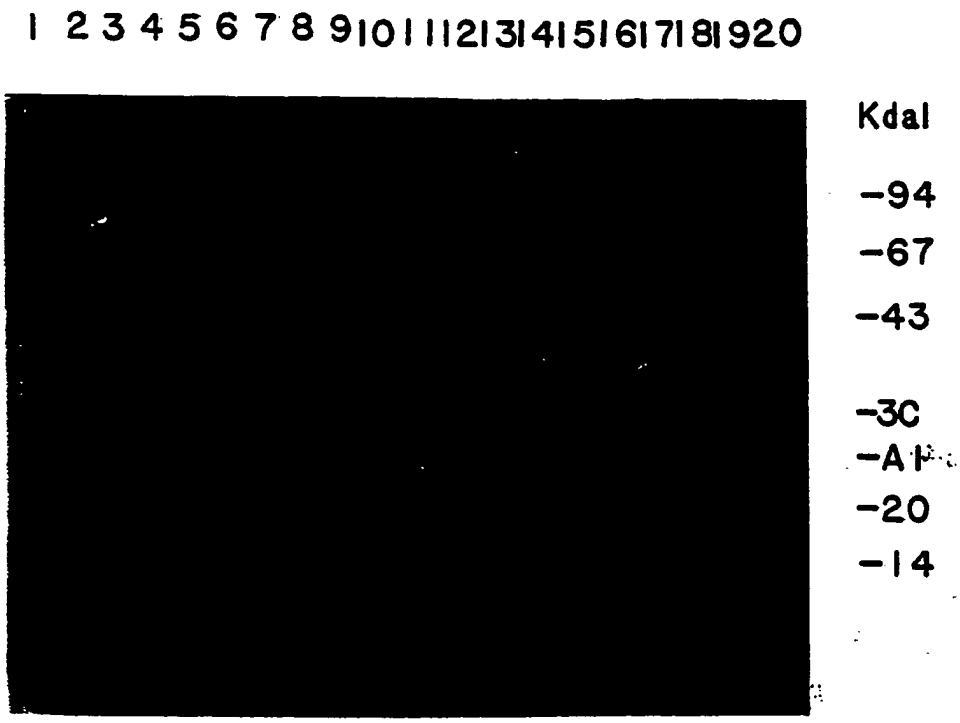


FIG. 15

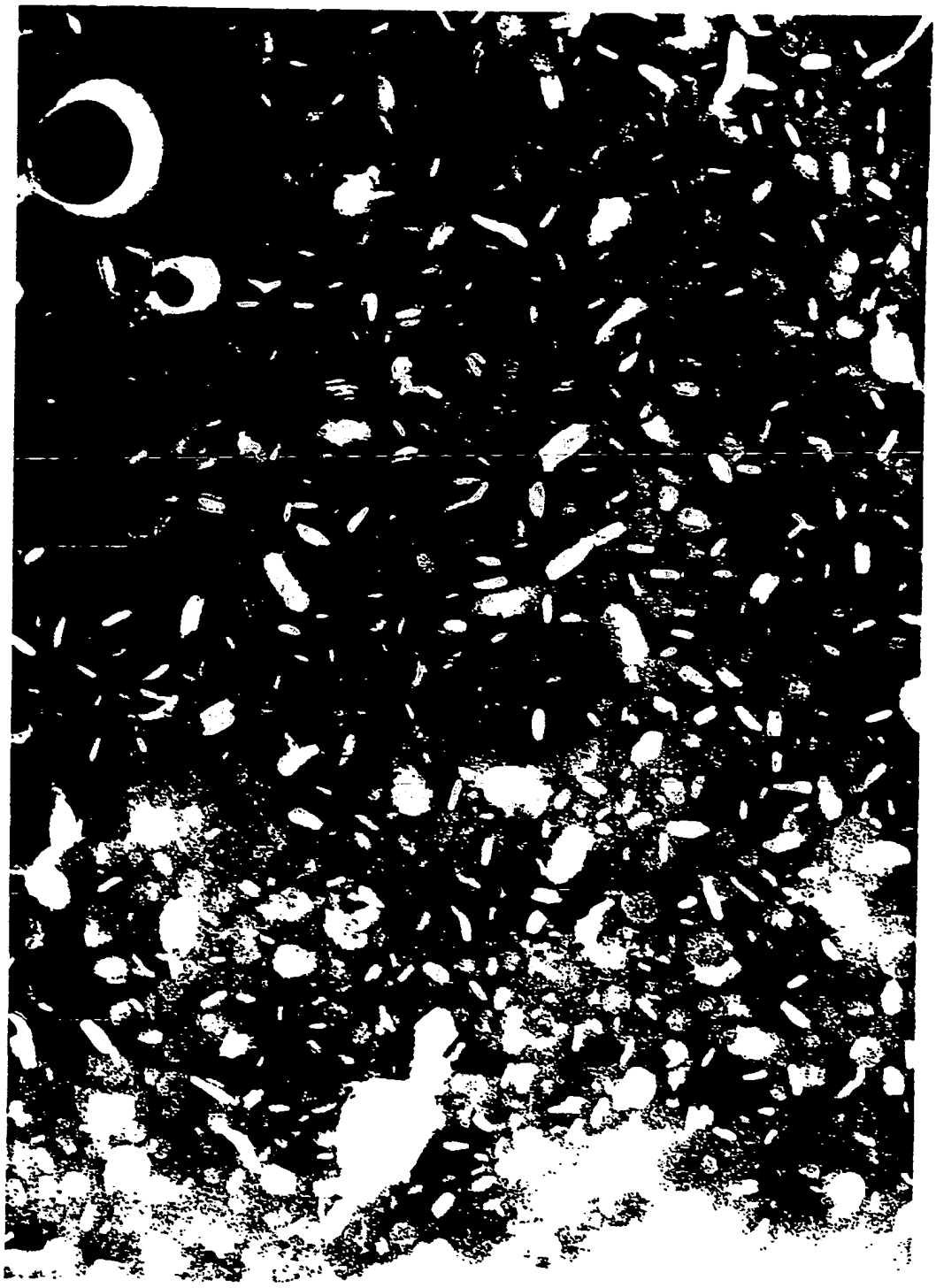


FIG. 16

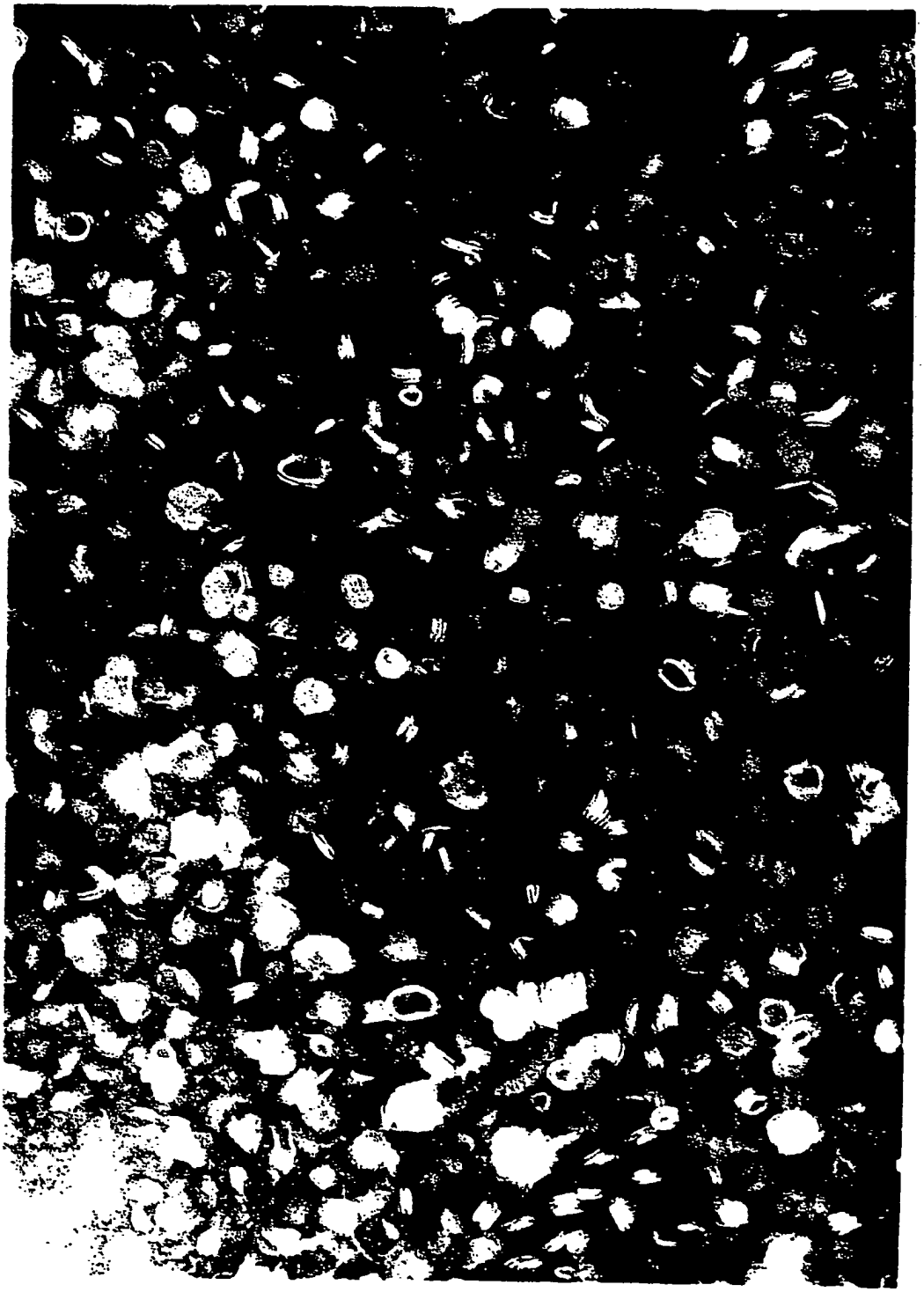


FIG. 17

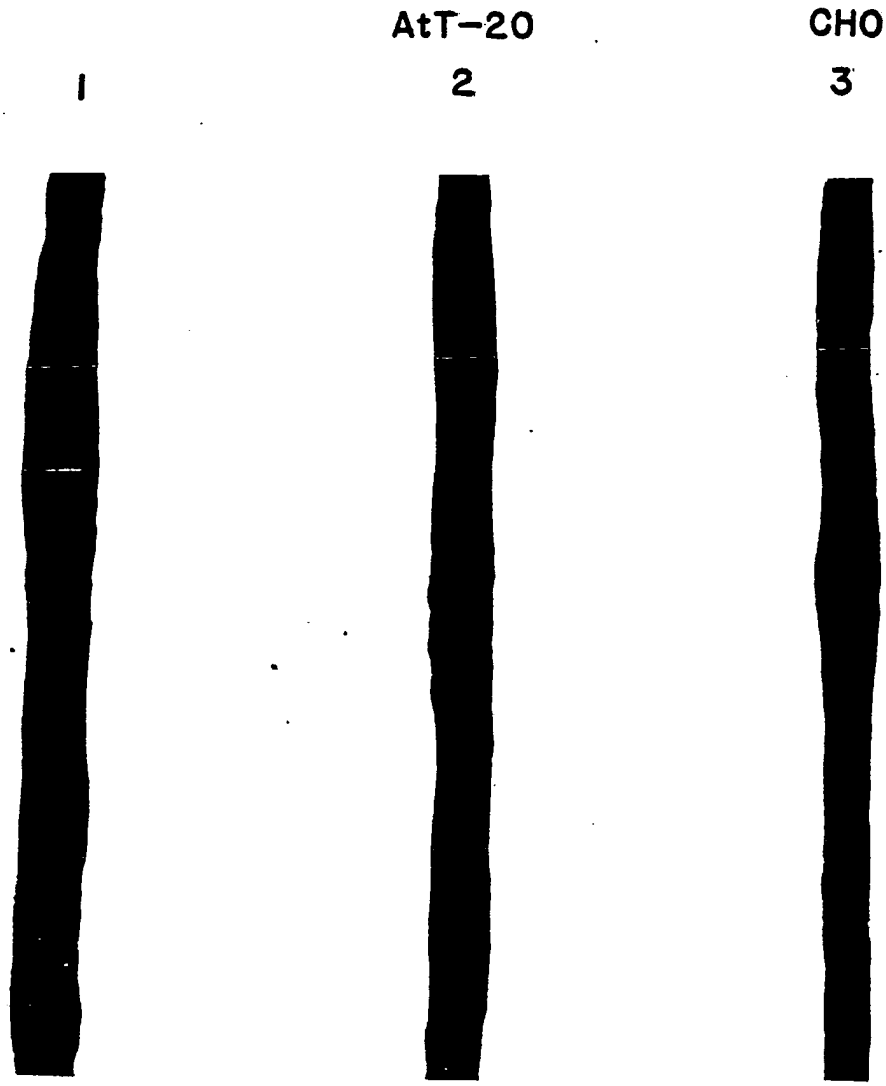


FIG. 18

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00296

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C12N 5/00; C07K 13/00; C07K 15/08

II. FIELDS SEARCHED

Minimum Documentation Searched [4]	
Classification System	Classification Symbols
U.S.	435/317; 935/71; 260/112R,112.5R; 435/68,70,91,172.3, 240,241,243,253; 948; 536/27; 935/9,10,11,12,13,14,27,32,34,36,66,70

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [5]

CA SEARCH DATABASE: 1967-1986

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	N, Karin et al, Nature, Vol. 299, 1982, pages 797-802.	1-5, 11-14
Y	N, Pavlakis et al, Proc. Natl. Acad. Sci. USA, Vol. 78, 1981, pages 7398-7402.	15
Y	N Pavlakis et al, Proc. Natl. Acad. Sci. USA, Vol. 80, 1983, pages 397-401.	1-5, 11-15
Y, P	N, Searle et al, Molec. Cell. Biol., Vol. 5, June 1985, pages 1480-1489.	1-5, 11-14
Y	N, Karin et al, Proc. Natl. Acad. Sci. USA, Vol. 80, 1983, pages 4040-4044.	2,3, 11-14
Y, P	N, White et al, Nature, Vol. 317, 1985, Pages 361-363.	16

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
23 April 1986	06 MAY 1986
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	James Martinell

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	N, Karin et al, Proc. Natl. Acad Sci. USA, Vol. 80, 1983, pages 4040-4044.	1,4,5
Y	U.S., A, 4,446,235, Published 1 May 1984, Seeburg et al.	15
Y	N, Hirano et al, Chemical Absracts, Vol. 97, 1982, Abstract No. 158949d of Nippon Sanka Fujinka Gakkol Zasshi, Vol. 34, 1982, pages 889-898.	16
Y	N, Berg et al, Molec. Cell Biol., Vol. 3, 1983, pages 1246-1254.	3-5

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	N, Richards et al, Cell, Vol. 37, 1984, pages 263-272.	1-5, 11-14
Y	N, Searle et al, Molec. Cell. Biol., Vol. 4, 1984, pages 1221-1230.	1-5, 11-14
Y	N, Karin et al, Nature, Vol. 308, 1984, pages 513-519.	1-5, 11-14

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [ ] Claim numbers ..... because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. [X] Claim numbers 6-10 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

Claims 6-10 are unsearchable because claim 6 recites, "the system of claims 1-5", and claim 7 recites "the cells of claims 1-6". As written and understood claim 6 depends from all of claims 1-5 and claim 7 depends from all of claims 1-6 making claims 6 and 7 improper multiple dependent claims. Claims 8-10 depend ultimately from claims 6 and 7 and are hence improper multiple dependent claims.

VI. [X] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-5 and 11-14 drawn to cells
II. Claim 15 drawn to growth hormone
III. Claim 16 drawn to pulmonary surfactant apoprotein

1. [X] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[X] No protest accompanied the payment of additional search fees.